

## Detection and Identification of *Leptospira interrogans* Serovars by PCR Coupled with Restriction Endonuclease Analysis of Amplified DNA

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Primers for PCR were selected from a sequenced fragment of clone pL590, which contains a repetitive element present in the genome of *Leptospira interrogans* serovar hardjo type hardjoprajitno (M. L. Pacciarini, M. L. Savio, S. Tagliabue, and C. Rossi, *J. Clin. Microbiol.* 30:1243–1249, 1992). A specific DNA fragment was amplified from the genomic DNAs of serovar hardjo type hardjoprajitno and nine serovars also belonging to *L. interrogans* as a consequence of the spread of the same or a closely related repetitive element within this species (Pacciarini et al., *J. Clin. Microbiol.* 30:1243–1249, 1992). In addition, specific amplification was obtained from two *Leptospira borgpetersenii* serovars (tarassovi and hardjo type hardjobovis). Negative PCR results were observed with all of the other *Leptospira* serovars tested, including nonpathogenic ones (serovars patoc and andamana), another spirochete (*Borrelia burgdorferi*), bacteria commonly found in biological samples, and swine and bovine cell lines. Direct PCR on biological samples such as kidney samples demonstrated that preliminary isolation and culture of *Leptospira* cells are not required for efficient detection. Furthermore, digestion of the amplified DNA with the enzymes *HinfI* and *DdeI* yielded specific polymorphic patterns, allowing discrimination among the majority of the serovars. These methods were applied to 25 field isolates of serovar pomona, leading to the conclusion that they were suitable for the simple and rapid detection of *L. interrogans* and for serovar identification.

Leptospirosis is a disease that occurs worldwide and that affects wild and domestic animals as well as humans (1). The taxonomy of the pathogenic leptospires was recently reorganized, on the basis of studies on DNA homology and DNA polymorphic patterns (24, 25, 44), into seven species comprising over 200 serovars (13). Many serovars are known to have preferential animal reservoirs or to be associated with a particular clinical form of infection (17). Therefore, simple detection of leptospires is usually inadequate for diagnostic and epidemiological purposes if it is not accompanied by serovar identification. Conventionally, these goals are met by culture isolation and serological techniques. Culture is slow, laborious, and susceptible to contamination, while serology does not provide reliable information on the carrier or shedding state in subjects with chronic infections and is unsatisfactory for rapid serovar characterization. Furthermore, because of extensive genetic heterogeneity, some serovars occur in more than one of the newly formed species (e.g., serovar hardjo, which is found both in *Leptospira interrogans* and in *Leptospira borgpetersenii*). Some of these drawbacks have been overcome by the introduction of molecular biology methods; restriction endonuclease analysis (7, 15, 27, 32, 33, 37, 38), Southern blot analysis (22, 23, 39, 41, 46), and pulsed-field gel electrophoresis (9, 10) have shown their utility for serovar identification; dot blot and in situ hybridization analyses (18, 34–36, 41, 45) have been used for the detection of leptospires in biological samples. However, these techniques are not readily applicable to routine work in diagnostic laboratories. Recently, the search for alternative methods has focused on specific DNA amplification by PCR. This technique has been

demonstrated to be sufficiently sensitive and rapid to be applied for the detection and characterization of microorganisms in blood and urine samples (5, 21).

The complexity of the *Leptospira* genus (4, 14, 20, 24, 25, 44) raises the issue of what should be the appropriate target for amplification by PCR; detection with primers for well-conserved sequences shared by all leptospires has been described (16). Examples of the opposite approach, with highly specific primers which allow amplification of sequences within only a certain serovar, also exist (8, 40, 43).

We found that distinctive repetitive elements were present within a single or a few *Leptospira* species and that their distributions were correlated with the degree of genetic relatedness among serovars (22). Thus, in theory, each repetitive element could be the target for species-specific PCR assays; classification could then follow on the basis of sequence polymorphisms among serovars. This rationale was applied in the present study to the detection and characterization of serovars belonging to *L. interrogans*.

Our data suggest that the combination of PCR and restriction enzyme analysis of the amplified products (PCR-restriction fragment length polymorphism [RFLP] analysis) can be used as a tool for a more informative diagnosis as well as for large-scale epidemiological studies.

### MATERIALS AND METHODS

**Bacterial strains, cell lines, and growth conditions.** The leptospiral serovars used in the present study are listed in Table 1 and are part of the collection of the Istituto Zooprofilattico Sperimentale of Brescia. In addition, 25 *Leptospira* isolates belonging to serovar pomona were obtained from a swine kidney in the course of the study and were characterized by Southern blot and monoclonal antibody analyses (30).

Culture conditions for the leptospires were essentially those

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TABLE 1. *Leptospira* strains used in the study

Serovar	Strain	Species <sup>a</sup>
Australis	Ballico	<i>L. interrogans</i>
Bataviae	Pavia 1	<i>L. interrogans</i>
Bratislava	Riccio 2	<i>L. interrogans</i>
Canicola	Alarik	<i>L. interrogans</i>
Copenhageni	Wijnberg	<i>L. interrogans</i>
Hardjo	Hardjoprajitno	<i>L. interrogans</i>
Icterohaemorrhagiae	Bianchi	<i>L. interrogans</i>
Lora	Riccio 37	<i>L. interrogans</i>
Pomona	Mezzano I	<i>L. interrogans</i>
Zanoni	Zanoni	<i>L. interrogans</i>
Castellonis	Castellon 3	<i>L. borgpetersenii</i>
Hardjo	Hardjobovis/Sponselee	<i>L. borgpetersenii</i>
Javanica	Veldrat Batavia 46	<i>L. borgpetersenii</i>
Mini	Sari	<i>L. borgpetersenii</i>
Saxkoebing <sup>b</sup>	Mus 24	<i>L. borgpetersenii</i>
Tarassovi	Mitis Johnson	<i>L. borgpetersenii</i>
Cynopteri	3522 C	<i>L. kirschneri</i>
Grippotyphosa <sup>c</sup>	Moskva V	<i>L. kirschneri</i>
Gorgas	1413 U	<i>L. santarosai</i>
Shermani	LT 821	<i>L. santarosai</i>
Celledoni	Celledoni	<i>L. weilii</i>
Andamana	CH 11	<i>L. biflexa</i>
Patoc	Patoc 1	<i>L. biflexa</i>

<sup>a</sup> Subdivision in species as proposed by Yasuda et al. (44) and Ramadass et al. (25).

<sup>b</sup> Saxkoebing is classified as *L. interrogans* by Yasuda et al. (44).

<sup>c</sup> Grippotyphosa is classified as *L. interrogans* by Yasuda et al. (44).

described by Johnson and Harris (11). *Borrelia burgdorferi* was grown at 30°C in BSKII medium (2). *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Streptococcus uberis* were grown in LB broth (19). Mammalian cell lines (IBRS2, Aubek, PK15) were propagated in Eagle's minimum essential medium to give a confluent monolayer in a T-25 flask.

**DNA extraction, cloning, and sequencing.** The preparation of plasmid and leptospiral genomic DNAs has been described previously (22). DNAs from other microorganisms or cell lines were extracted by standard procedures (28). Isolation of plasmid pL590 from a genomic library of serovar hardjo strain Hardjoprajitno has been reported previously (22).

The 622-bp *AccI-EcoRI* fragment e of pL590 was subcloned in the vector pBluescriptII KS+ purchased from Stratagene; purified single-stranded DNA was prepared as described in the Stratagene manual and was sequenced by the method developed by Sanger and coworkers (29) by using the Sequenase version 2.0 kit (United States Biochemicals).

**Preparation of samples for PCR analysis. (i) Pure leptospire.** Cultured leptospire were counted microscopically in a Burkner chamber, and serial 10-fold dilutions (from 10<sup>6</sup> to 1 cell) were made in a total volume of 20 µl of sterile distilled water. Samples were then boiled for 10 min and quickly chilled on ice.

**(ii) Reconstitution experiments with biological samples.** Serial 10-fold dilutions of cultured leptospire (from 5 × 10<sup>8</sup> to 5 × 10<sup>2</sup> cells) were mixed with 5 mg of kidney collected from a healthy swine. Each sample was homogenized for a few seconds in an Ultra-Turrax type 18-10 homogenizer (IKA-WERK, Staufen, Germany) in the presence of 5 ml of phosphate buffered saline; 10-µl aliquots (containing a final concentration of 10<sup>6</sup> to 1 cell) were submitted to the PCR after a short centrifugation step and boiling treatment as described by Bélač et al. (3).

PCR of pomona isolates was performed on 10 µl of *Lepto-*

*spira* cultures at a final concentration of about 2.5 × 10<sup>8</sup> cells per ml or on 10 µl of the homogenized kidney samples treated as described above.

**PCR assay and hybridization experiments.** In order to achieve the best sensitivity for the PCR, several combinations of annealing temperatures (from 50 to 60°C), number of cycles (from 25 to 30), and concentrations of MgCl<sub>2</sub> (from 0.5 to 3.0 mM) and primers (from 0.2 to 1 µM) were tested. The following conditions were chosen: PCR was performed in a total volume of 100 µl containing 10 µl of 10× reaction buffer (500 mM KCl, 15 mM MgCl<sub>2</sub>, 100 mM Tris-HCl [pH 8.3]), primers (final concentration, 1 µM), the four deoxynucleotide triphosphates (Pharmacia) at a final concentration of 200 µM each, and 2.5 U of *Taq* polymerase (Perkin-Elmer). The samples were covered with a drop of mineral oil and placed in an automatic PCR thermal cycler (Perkin-Elmer). One amplification cycle consisted of annealing of primers for 1 min at 55°C, elongation for 1.5 min at 72°C, and denaturation for 1 min at 95°C. Thirty amplification cycles were used, and the last elongation step was extended to 10 min.

The sequences of primers 590-dir1 and 590-rev2 are underlined in Fig. 2.

After amplification, a 20-µl portion of each sample was subjected to electrophoresis on a 1.5% agarose gel. Gels were photographed and blotted to activated nylon membrane (Zetaprobe; Bio-Rad) by the method described by Southern (31).

Dot blot experiments were performed on 20-µl aliquots of the PCR mixtures by using a Minifold apparatus (Schleicher & Schuell) following the procedure described by Kafatos et al. (12).

Filters were hybridized with the pL590 fragment e or with the entire pL590 plasmid. Labelling and hybridization conditions have been described previously (22).

**Restriction endonuclease analysis of PCR-amplified products.** Aliquots (20 to 40 µl) from the PCR mixtures were diluted to a final volume of 200 µl and were digested with the restriction enzymes *AluI*, *AflIII*, *BspHI*, *DdeI*, *DpnI*, *FokI*, *HinfI*, *MboI*, *MboII*, *SauI*, and *Sau3A* according to the instructions of the supplier (Boehringer, Mannheim, Germany). After digestion, the PCR samples were ethanol precipitated and centrifuged at 12,000 × g for 30 min at 4°C, and the resulting pellet was resuspended in 20 µl of TE (10 mM Tris-HCl, 1 mM EDTA [pH 8]). The DNA fragments were fractionated by electrophoresis for 6 h at 80 V in a 4% Nusieve agarose gel (FMC) buffered with TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and stained with ethidium bromide.

**Nucleotide sequence accession number.** The DNA sequence data reported here have been assigned GenBank accession number X58829.

## RESULTS

**Distribution of a cloned repetitive element among *Leptospira* serovars.** Fragment e was subcloned from plasmid pL590 and contains a portion of a repetitive element that was present in at least nine copies in the hardjoprajitno genome as determined by Southern hybridization analysis (22).

The presence of the same or closely related sequences was assessed in 23 *Leptospira* serovars representative of six *Leptospira* species (Table 1), including the nonpathogenic *Leptospira biflexa*.

As expected from previous hybridization studies performed with clone pL590 (22), fragment e hybridized extensively under high-stringency conditions to the DNAs of *L. interrogans* strains from serovars australis, bratislava, lora, hardjo type

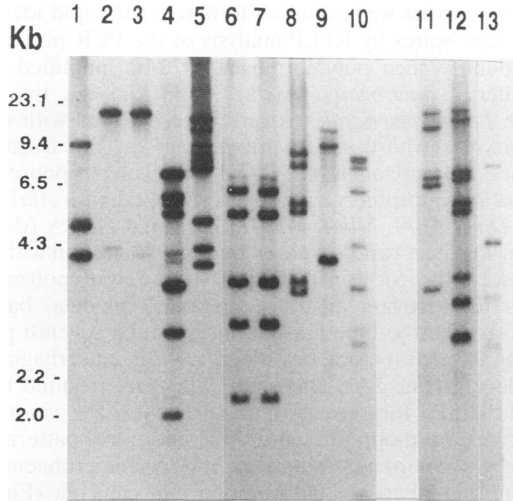


FIG. 1. Hybridization patterns of *Bgl*II-digested chromosomal *Leptospira* DNAs probed with fragment e under high-stringency conditions. Lanes: 1, australis; 2, bratislava; 3, lora; 4, hardjoprajitno; 5, pomona; 6, icterohaemorrhagiae; 7, copenhageni; 8, bataviae; 9, zanoni; 10, canicola; 11, tarassovi; 12, hardjobovis; 13, celledoni. The numbers on the left correspond to the length (in kilobases) of *Hind*III-digested bacteriophage lambda DNA marker.

hardjoprajitno, pomona, icterohaemorrhagiae, copenhageni, bataviae, zanoni, and canicola (Fig. 1, lanes 1 through 10, respectively). No hybridization, even after long exposure times, was detected for the other *Leptospira* species when strains of serovars castellonis, javanica, mini, saxkoebing, grippotyphosa, shermani, gorgas, cynopteri, and the nonpathogenic serovar patoc were used (data not shown). The only exceptions were two *L. borgpetersenii* serovars (tarassovi and hardjo type hardjobovis), which gave a strong hybridization signal, and the *Leptospira weilii* serovar celledoni, which hybridized to a lesser extent (Fig. 1, lanes 11, 12, and 13).

**Sequence analysis of fragment e.** Hybridization experiments with the complete pL590 clone and several subclones (fragments d, e, and f) led to the conclusion that fragment e exists entirely within the repetitive element (22) (data not shown). The repetitive element extends for a few nucleotides upstream of fragment e into fragment d and for at least 200 nucleotides downstream into fragment f. The sequence of fragment e was determined (Fig. 2) and was confirmed by sequencing of an independent hardjoprajitno clone also containing the same repetitive element (22a). The sequence was 622 bp long, with a 40% G+C content, which is slightly greater than the mean value of 35.5% reported for *L. interrogans* (44). No significant open reading frame was present, with the exception of one of 99 amino acids in length starting at nucleotide 35 of the complementary strand. A computer search of DNA and protein databases (PCgene version 6.6; Intelligenetics) did not reveal any significant similarity to known sequences. The fragment e sequence was used to design the 590-dir1 and 590-rev2 primers (highlighted in Fig. 2) for use in the PCR and as the basis for the choice of the restriction enzymes that should be tested in order to obtain polymorphic restriction patterns.

**Specificity and sensitivity of PCR amplification.** After optimization of the parameters of the PCR assay with genomic DNA from strain Hardjoprajitno as the target for amplification, the same parameters were applied to aliquots (1 ng) of

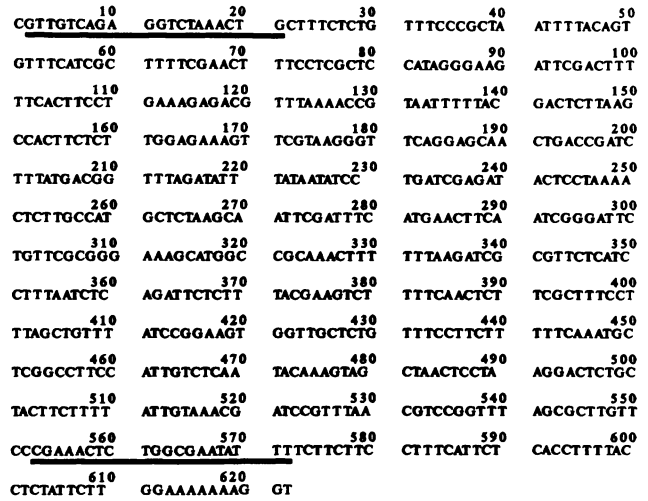


FIG. 2. Nucleotide sequence of fragment e derived from a genomic library of *L. interrogans* serovar hardjo type hardjoprajitno. The sequences corresponding to the oligonucleotide primers 590-dir1 (upstream) and 590-rev2 (downstream) are underlined.

genomic DNA purified from the strains listed in Table 1. Strains of serovars australis, bratislava, lora, pomona, icterohaemorrhagiae, copenhageni, bataviae, canicola, zanoni, tarassovi, and hardjo (type hardjobovis) yielded one or two closely migrating bands of about 570 bp, as expected (Fig. 2). These bands were easily detectable on ethidium bromide-stained agarose gels (see Fig. 5C; data not shown). Amplification of DNAs from these strains was in agreement with the presence of bands that strongly hybridized to fragment e in the Southern blot experiments. Strains belonging to serovars castellonis, javanica, mini, saxkoebing, cynopteri, grippotyphosa, gorgas, and shermani did not yield any amplified DNA, again in agreement with the results of Southern blot experiments. Interestingly, no amplification was detected with DNA from serovar celledoni, despite the weak hybridization detectable on Southern blots.

To evaluate the specificities of the primers, we performed PCRs under conditions identical to those described above but with different amounts of genomic DNA (1, 10, 100, and 1,000 ng) from various organisms; no amplification was detected with DNAs from bacteria related to *L. interrogans* such as the nonpathogenic *L. biflexa* (serovar andamana) and *B. burgdorferi* or from more evolutionarily distant organisms such as *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, *S. aureus*, or *S. uberis* (data not shown). Amplification of DNAs from swine and bovine cell lines resulted in several aspecific products which became visible at the highest concentration of starting DNA (1,000 ng). PCR of as little as 10 ng of DNA from *Salmonella typhimurium* and *L. biflexa* serovar patoc yielded bands of 1,400 and 580 bp, respectively; however, these bands did not hybridize to the hardjoprajitno-specific probe pL590 (data not shown).

The sensitivity of the PCR was evaluated by gel electrophoresis and dot blot hybridization of the amplified DNA. Dot blot hybridizations with probe pL590 showed that as little as  $10^{-5}$  ng of starting DNA (corresponding to 1 to 10 cells) could be detected from strain Hardjoprajitno and from the related serovars pomona, canicola, bataviae, copenhageni, and icterohaemorrhagiae; weaker signals were obtained from serovars australis, zanoni, tarassovi, and hardjo type hardjobovis. Tenfold greater amounts of starting DNA ( $10^{-4}$  ng) were neces-

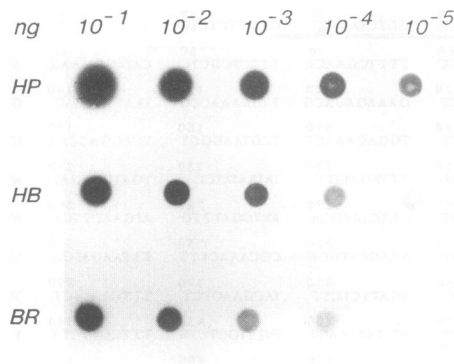


FIG. 3. Dot blot hybridization of aliquots from the mixture for the PCR performed on serial dilutions of hardjoprajitno (HP), hardjobovis (HB), and bratislava (BR) DNAs (from  $10^{-1}$  to  $10^{-5}$  ng) and hybridized to fragment e.

sary to give visible hybridization for serovars bratislava and lora. As a representative example, Fig. 3 shows the dot blot results of serovars hardjo type hardjoprajitno, hardjo type hardjobovis, and bratislava. Analysis by agarose gel electrophoresis required 10-fold more starting DNA than dot blot hybridization to yield a visible band (data not shown).

**Direct amplification from *Leptospira* cells and biological samples.** In order to make PCR amenable to the analysis of large numbers of samples, it would be desirable to bypass the need to isolate *Leptospira* cells or purify genomic DNA. This goal was achieved by subjecting *Leptospira* cells to boiling (see Materials and Methods) prior to the standard PCR. Agarose gel electrophoresis of the amplified DNA from hardjoprajitno cells revealed the predicted band for samples containing  $10^6$  to  $10^2$  leptospires. The sensitivity was further improved by dot blot hybridization, allowing detection of one or a few cells (data not shown). The performance of the PCR assay on biological samples was tested by seeding fragments of kidney collected from a healthy swine with known amounts of leptospires. The samples were treated as described in Materials and Methods, subjected to PCR, and analyzed by agarose gel electrophoresis. Several aspecific bands were present after ethidium bromide staining; nevertheless, a clear and specific hybridization signal was obtained by Southern blot analysis of 20- $\mu$ l aliquots from samples containing from  $10^6$  to 10 leptospires (Fig. 4).

**Serovar differentiation through restriction patterns of PCR products.** The natural variability among DNA sequences of

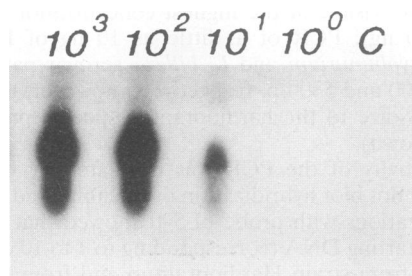


FIG. 4. Southern blot hybridization of PCR products from kidney samples containing serial dilutions (from  $10^3$  to  $10^0$ ) of *L. interrogans* serovar hardjo type hardjoprajitno cells. C, kidney sample from a healthy swine. The probe was radiolabelled fragment e.

different serovars was exploited to obtain the rapid identification of leptospires by RFLP analysis of the PCR products.

To identify such polymorphisms, 570-bp amplified DNAs from the *L. interrogans* serovars and from the two PCR-positive *L. borgpetersenii* serovars were digested with restriction enzymes whose sites were present on the fragment e sequence and were analyzed by agarose gel electrophoresis. No or a few polymorphic variations were found with *AluI*, *AflII*, *BspI*, *DpnI*, *FokI*, *MboI*, *MboII*, *SauI*, and *Sau3A* (data not shown). The best results were obtained by digestion with *HinI* and *DdeI* (Fig. 5). *HinI* yielded six different polymorphic profiles for serovars australis, pomona, canicola, bataviae, zanoni, and hardjo type hardjoprajitno and a seventh pattern common to serovars lora, bratislava, icterohaemorrhagiae, and copenhageni (Fig. 5A). Digestion with *DdeI* resulted in four distinct RFLPs for serovars australis, bataviae, zanoni, and hardjo type hardjoprajitno and three additional patterns each shared by two serovars: bratislava and lora, icterohaemorrhagiae and copenhageni, and pomona and canicola (Fig. 5B). Finally, two new restriction patterns were obtained by digestion with *HinI* and *DdeI* of DNAs from the *L. borgpetersenii* serovars tarassovi and hardjo type hardjobovis (Fig. 5C).

The sizes of the digested fragments were expected to sum to that of the amplified band (570 bp). While this was true for most serovars, exceptions were also noted, e.g., *HinI* digestion of serovars zanoni and pomona (Fig. 5A, lanes 7 and 10) and *DdeI* digestion of hardjo type hardjoprajitno (Fig. 5B, lane 11). This fact could be explained by the occurrence of sequence polymorphisms among multiple copies of the repetitive element present within the genome. Such variability also affected the size of the amplified product, as exemplified by the two closely migrating bands from PCR of hardjo type hardjoprajitno DNA (Fig. 5C, lane 10). Potential technical artifacts caused by PCR or partial digestion of DNA were excluded by Southern blot hybridization with the fragment e probe (data not shown) and by multiple restriction enzyme digestions at different times with many independent samples.

Since the ultimate goal of our experiments was to apply PCR and RFLPs of the amplified fragments to the characterization of field isolates, it was important to show that the results obtained with the laboratory strains were reproducible with clinical samples. For this purpose, 25 independent field samples were previously analyzed by measuring their immunoreactivities with monoclonal antibodies and Southern blot analysis and were found to belong to serovar pomona (30). Cultures of each isolate were then subjected to boiling, PCR, and restriction enzyme analysis. DNAs from all the 25 isolates could be amplified and, after digestion with *HinI* and *DdeI*, yielded patterns identical to those of the pomona Mezzano I reference strain (data not shown).

Furthermore, direct detection and identification were attempted on fragments of kidney tissue taken from 3 of the 25 swine which were positive for serovar pomona. Similar to the reconstitution experiment (see above), digestion of the amplified DNA with *HinI* and *DdeI* produced a large number of bands because of nonspecific DNA amplification; these bands were resolved in a clear pattern, identical to the one from pure serovar pomona cell cultures, after Southern blot and hybridization with fragment e (Fig. 6) (data not shown).

## DISCUSSION

Several studies have applied PCR to the amplification of *Leptospira* DNA by using either well-conserved primers, selected within the sequence of the *Leptospira* rRNA 16S gene (16), or primers which are specific for one or a few serovars (8,

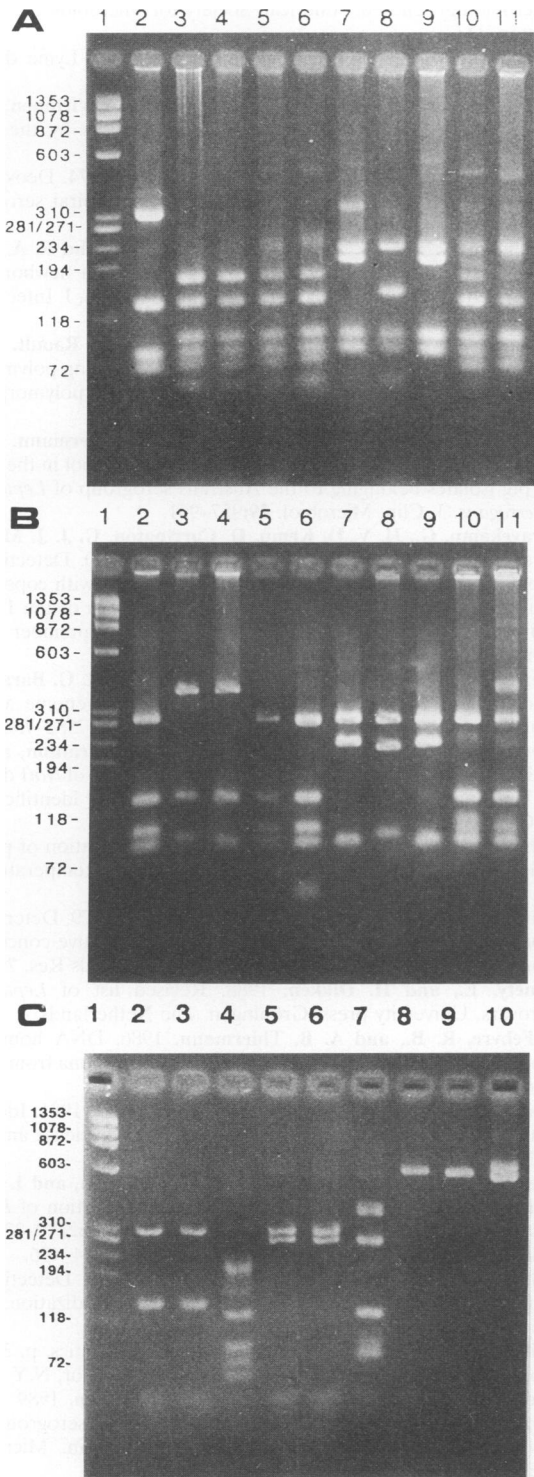


FIG. 5. (A and B) Gel electrophoresis performed in 4% Nusieve (FMC) agarose gel of PCR-amplified products from serovars australis (lane 2), bratislava (lane 3), lora (lane 4), icterohaemorrhagiae (lane 5), copenhageni (lane 6), pomona (lane 7), bataviae (lane 8), canicola (lane 9), zannoni (lane 10), and hardjo type hardjoprajitno (lane 11). The samples were digested with either *Hinf*I (A) or *Dde*I (B). (C) Gel electrophoresis performed in a 4% Nusieve (FMC) agarose gel of PCR products from serovars hardjo type hardjobovis (lanes 2, 5, and 8), tarassovi (lanes 3, 6, and 9), and hardjo type hardjoprajitno (lanes 4, 7, and 10). The samples were digested with *Hinf*I (lanes 2, 3, and 4) or

40, 43). We were interested in the development of PCR assays whose target was broad and yet confined to a single *Leptospira* species. This approach should be more informative, for diagnostic purposes, than those based on the detection of rRNA genes, which leads to the amplification of DNAs from most *Leptospira* serovars, including nonpathogenic ones. At the same time, the possibility of detecting all of the *L. interrogans* serovars with just two primers was viewed as more practical than the use of serovar-specific primers, because in this case a large number of independent reactions would be needed to analyze each clinical sample.

After detection of a *Leptospira* infection, it is often necessary, for diagnostic and epidemiological purposes, to identify the serovar involved; therefore, the existence of RFLPs among the amplified DNAs was sought. Similar PCR-RFLP strategies were previously applied to the classification of other bacteria (6, 26), and while the present study was in progress, a report describing the differentiation of *Leptospira* serovars by PCR and RFLP of the endoflagellin gene (42) was published.

The primers 590-dir1 and 590-rev2 were designed to amplify fragment e, which contains the majority of a novel repetitive sequence detected by Southern blot hybridization in all of the *L. interrogans* serovars tested but not in other *Leptospira* species (22; this work), with the exception of two *L. borgpetersenii* serovars (hardjo type hardjobovis and tarassovi). A third serovar from *L. weilii* hybridized weakly, and its DNA could not be amplified by PCR. In principle, the multicopy nature of the fragment e sequence could provide the following two significant advantages for PCR-RFLP: an enhanced sensitivity of PCR because of the availability of more than one target per genome and better chances of finding sequence and length polymorphisms. The first prediction was confirmed by routine detection of one or a few *Leptospira* cells, with the exception of serovars lora and bratislava, which contain a single copy of the element and require 10-fold more starting material (compare Fig. 1 and 3). The occurrence of sequence and length polymorphisms among serovars was also verified. Such polymorphisms were found even within the same serovar, as exemplified by hardjo type hardjoprajitno, from which two products of slightly different sizes could consistently be amplified. Southern blot experiments and sequencing demonstrated that both products derived from the repetitive element, the only difference among them being a 13-bp insertion within the reported fragment e sequence (22a). Sequence polymorphisms of the repetitive element were detected with several restriction enzymes; among them *Dde*I and *Hinf*I gave the most informative patterns, allowing us to differentiate most of the serovars. It should be pointed out that any ambiguity was just between two serovars (e.g., between serovars lora and bratislava or serovars icterohaemorrhagiae and copenhageni), and the presence of identical restriction patterns correlated very well with the overall genetic similarities of the serovars involved.

As demonstrated by the data gathered on the specificity and reproducibility of the PCR-RFLP method, its application to field isolates may be feasible. Bacteria that commonly contaminate *Leptospira* samples and *Leptospira* cells not containing the repetitive element were always found to be negative even when PCR was primed with amounts of DNA orders of magnitude higher than that required for efficient amplification from positive cells. Amplification in the presence of animal cell

*Dde*I (lanes 5, 6, and 7) or were left undigested (lanes 8, 9, and 10). *Hae*III-digested bacteriophage  $\phi$ X174 DNA marker (Boehringer) was loaded onto the first lane of each gel.

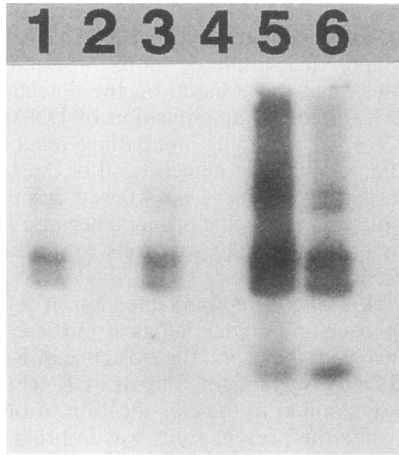


FIG. 6. Southern blot hybridization of PCR products from three swine kidney samples infected with serovar pomona (lanes 1, 3, and 5). DNA was digested with *DdeI* and hybridized to fragment e. Lanes 2 and 4, PCR products of kidney samples from noninfected animals; lane 6, PCR product from the pomona reference strain Mezzano I.

DNAs resulted in the synthesis of unspecific products because of the complexity of the mammalian genome. This background was easily eliminated by a hybridization step with fragment e as the probe. This also applied to direct amplification from tissues (kidney) infected with *Leptospira* cells. In addition, the sensitivities of direct PCR on tissues and of PCR on pure *Leptospira* cultures were comparable, as shown by the results of the reconstitution experiments.

Evidence that the results with the laboratory strains were reproducible on field isolates was provided by carrying out PCR-RFLP on 25 serovar pomona isolates obtained at different times and from different geographic locations and previously characterized by Southern blot hybridization and monoclonal antibody reactivities (30). It will be necessary to analyze more serovars and to keep a collection of patterns for field isolates, because we anticipate that new distinctive restriction patterns will be found because of the inherent variability of the repetitive element.

The repetitive element appeared to be ubiquitous within the *L. interrogans* species, but it could also be amplified from two *L. borgpetersenii* serovars. This did not constitute a drawback since the *L. borgpetersenii* serovars showed a characteristic RFLP.

The work described here represents a step toward the development of a universal diagnostic PCR for *Leptospira* species that is based on the use of a limited number of primers and then RFLP analysis for serovar identification. We were encouraged to pursue this goal by the finding (22a) of other repetitive elements that, like fragment e, are restricted to one or a few *Leptospira* species.

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