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Repetitive Sequences Cloned from *Leptospira interrogans* Serovar hardjo Genotype hardjoprajitno and Their Application to Serovar Identification

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We selected, from a genomic library of *Leptospira interrogans* serovar hardjo genotype hardjoprajitno, two probes containing repetitive sequences (pL1 and pL590). The hybridization patterns of these probes to DNA isolated from a variety of *Leptospira* serovars were examined and their ability to detect subtle differences at the genomic organization level was established. We identified the DNA fragments within pL1 and pL590 which are sufficient to yield polymorphic hybridization patterns; these results define the upper size limit of two novel repetitive elements in the *Leptospira* genome. The pattern and degree of hybridization observed for the serovars tested in this work were used to divide *Leptospira* spp. into groups which share genetic relatedness; our conclusions are consistent with previous classifications by other authors.

Leptospirosis is an important disease of livestock caused by *Leptospira interrogans*. This species is immunologically complex and comprises 212 serovars organized into 23 serogroups (7) on the basis of their antigenic relatedness. Conventional serogroup and serovar identification of the genus *Leptospira* is performed by microscopic agglutination and cross-absorption tests with group- and serovar-specific hyperimmune sera. Such analysis is time-consuming and is often complicated by the presence of cross-reacting antigens between and within serogroups.

The use of monoclonal antibodies has improved various aspects of serovar identification (16, 18); nevertheless, this method cannot be applied to the detection of intraserovar differences that may be important in epidemiology or pathogenicity studies. For instance, the hardjobovis and hardjoprajitno genotypes share similar antigens and are both classified within serovar hardjo although they are genetically distinct and responsible for different clinical symptoms and epidemiology in cattle (2, 20).

For these reasons, several laboratories have developed restriction endonuclease analysis (REA) of genomic DNA as an alternative method for classifying *L. interrogans* (9, 12, 15, 17, 20, 22). A valuable addition to REA is represented by Southern blot analysis with specific DNA probes (25, 26, 29). This technique overcomes some limitations of REA, such as the interpretation of complex DNA banding patterns (25), the lack of discrimination between some genetically similar serovars (29), and the need to use relatively large amounts of purified DNA.

Various studies employing techniques based on genomic DNA annealing (8, 10, 24) have shown that *L. interrogans* contains a great deal of heterogeneity, leading to proposals for the existence of separate genetic groups within *L. interrogans* (1, 8, 10, 24).

The present work was conducted in order to isolate DNA probes that were suitable for identification and classification of *Leptospira* isolates by means of Southern blot hybridization to endonuclease-digested genomic DNA. A genomic library from *L. interrogans* genotype hardjoprajitno was constructed and, among several hundred clones isolated, we characterized two (pL1 and pL590) that contain novel repetitive sequences.

MATERIALS AND METHODS

Leptospiral strains. Some of the strains used in this study, listed in Table 1, were representative strains of the various serovars rather than reference strains; all of them were kindly supplied by M. Cinco, University of Trieste, except when noted. *Leptospira* strains designated 69 and 70 were field isolated from bovine kidneys (Istituto Zooprofilattico of Brescia). *Leptospira* cultures were grown at 30°C in EMJH medium (Difco Laboratories, Detroit, Mich.) for at least 1 week, essentially as described by Johnson and Harris (6).

DNA extraction. Exponentially growing leptospiral cultures were centrifuged at 12,000 × *g* for 30 min at 4°C. The resulting pellet was treated to extract genomic DNA as described by Marshall et al. (9); the amount and integrity of the DNA were evaluated by UV absorption and agarose gel electrophoresis. Plasmid DNA was prepared from *Escherichia coli* cells in 200-ml overnight cultures by using the alkaline lysis method and purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient (13).

Restriction endonuclease digestion and Southern blot hybridization of DNA. *Leptospira* DNAs (4 μg) were digested with *Bgl*II, *Hin*dIII, *Hae*III, *Pst*I, and *Eco*RI restriction endonucleases purchased from Boehringer (Mannheim, Germany) and used according to the specifications of the manufacturer. Digested DNA fragments were fractionated by electrophoresis overnight at 50 V in 0.7% agarose gels buffered with TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA).

Genomic DNA fragments were blotted to activated Nylon membrane (Bio-Rad) according to the method described by Southern (14), using 20× SSPE instead of 20× SSC as the transfer solution (1× SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4).

Recombinant plasmids and chromosomal DNA were labelled to a specific activity of at least 10^8 cpm/μg of DNA by random priming, using the Multiprime DNA labelling system kit (Amersham) according to the instructions of the manu-

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TABLE 1. Bacterial strains used in this study

Organism and serogroup	Serovar	Type and/or strain
L. interrogans		
Australis	Australis	Ballico
Australis	Bratislava	Riccio 2
Australis	Bratislava	SV 396
Australis	Lora	Riccio 37
Ballum	Castellonis	Castellon 3
Bataviae	Bataviae	Pavia 1
Canicola	Canicola	Alarik
Grippotyphosa	Grippotyphosa	Moskva V
Hebdomadis	Mini	Sari
Icterohaemorrhagiae	Icterohaemorrhagiae	Poletti
Javanica	Javanica	Veldrat Batavia 46
Pomona	Pomona	Mezzano I
Pyrogenes	Zanoni	Zanoni
Sejroe	Hardjo	Hardjoprajitno
Sejroe	Hardjo	Hardjobovis strain Sponselee
Sejroe	Saxkoebing	Mus 24
Shermani	Shermani	LT 821
Tarassovi	Tarassovi	Mitis Johnson
L. biflexa		
Semaranga	Patoc	Patoc 1

facturer. Prehybridization (4 h) and hybridization (overnight) were performed at 42°C in 50% formamide–750 mM NaCl–5× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone)–0.35 mg of salmon sperm DNA per ml–0.1% sodium dodecyl sulfate (SDS)–50 mM Na_2HPO_4/NaH_2PO_4 (pH 7)–10% dextran sulfate. After hybridization, the filters were washed once in 2× SSPE at room temperature and then twice in 0.1× SSPE–0.1% SDS at 65°C (high-stringency conditions) or at 42°C (low-stringency conditions).

Molecular cloning techniques. Purified chromosomal DNA of *L. interrogans* serovar hardjo genotype hardjoprajitno was digested to completion with the enzyme *Bgl*II, ligated to *Bam*HI-digested pUC8 plasmid (23), and used to transform *E. coli* JM101 cells. Colonies harboring recombinant plasmids (on the basis of isopropyl-β-D-galactopyranoside–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [IPTG-X-Gal] selection) were grown on nitrocellulose filters overlaying LB agar plates (LB is 1% Bacto Tryptone, 0.5% Bacto Yeast Extract, and 171 mM NaCl) supplemented with ampicillin (100 μg/ml).

Colony hybridization was performed according to the method of Grunstein and Hogness (5); the tracer used was chromosomal hardjoprajitno DNA labelled with [α-^{32}P] dCTP as described above. Plasmid DNAs were extracted by a small-scale rapid alkaline lysis method (13) and used to probe a Southern blot containing *Bgl*II digestions of hardjoprajitno genomic DNA. Isolation of individual DNA bands was achieved by extraction and purification from agarose gels, using Geneclean (Bio 101, Inc.) according to the instructions supplied with the kit.

RESULTS

Selection of genomic clones. Screening of the hardjoprajitno genomic library yielded several hundred recombinant clones. Twenty-two clones, selected on the basis of their strong autoradiographic signal after colony hybridization, were investigated. Twelve of them hybridized to only one or a few genomic bands when labelled and used as probes against hardjoprajitno DNA and were not further characterized. Comparison of the Southern blot banding patterns of the remaining clones indicates that they contained two distinct multicopy elements. We selected two recombinant plasmids, pL1 and pL590, representative of each element, with inserts of 3.2 and 5.4 kb, respectively. Figure 1 is an autoradiogram of a Southern blot of *Bgl*II-digested hardjoprajitno DNA run in parallel with purified inserts of pL1 and pL590 and probed with pL1 and pL590. This experiment shows the repetitive nature of these elements within the hardjoprajitno genome (20 to 13 bands of the indicated sizes are recognizable). As expected, the size of the cloned inserts corresponds to one of the genomic fragments, as shown by hybridization.

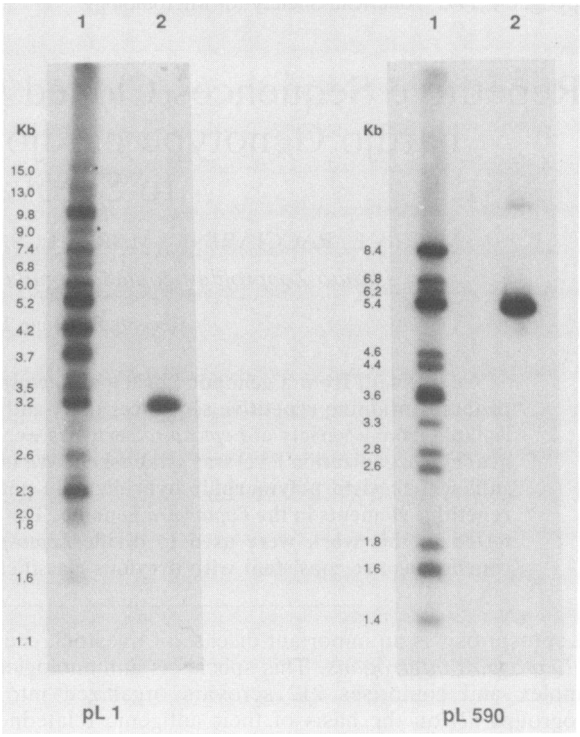

FIG. 1. Southern blot analysis of *Bgl*II-digested hardjoprajitno DNA (lanes 1) hybridized to pL1 and pL590. Lanes 2 contain 5 ng each of the pL590 and the pL1 inserts. The fragments were obtained by digesting pL1 with *Eco*RI and *Hin*dIII and pL590 with *Sal*I and *Sma*I and were purified by Geneclean (see Materials and Methods). Molecular sizes were calculated on the basis of lambda DNA *Hin*dIII-digested marker.

The probes did not hybridize to DNA from bacteria that may contaminate *Leptospira* isolates, such as *E. coli*, *Streptococcus uberis*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* (data not shown).

Identification of serovars by REA and Southern analysis. We performed Southern blot analysis under conditions of high stringency with DNA from 16 *Leptospira* serovars digested with *Eco*RI, *Pst*I, *Hin*dIII, and *Bgl*II restriction enzymes and hybridized with pL1 and pL590. Figure 2 shows the results for the restriction enzyme *Bgl*II. Several independent isolates from the majority of these serovars were analyzed in order to determine the reproducibility of the resulting patterns (data not shown).

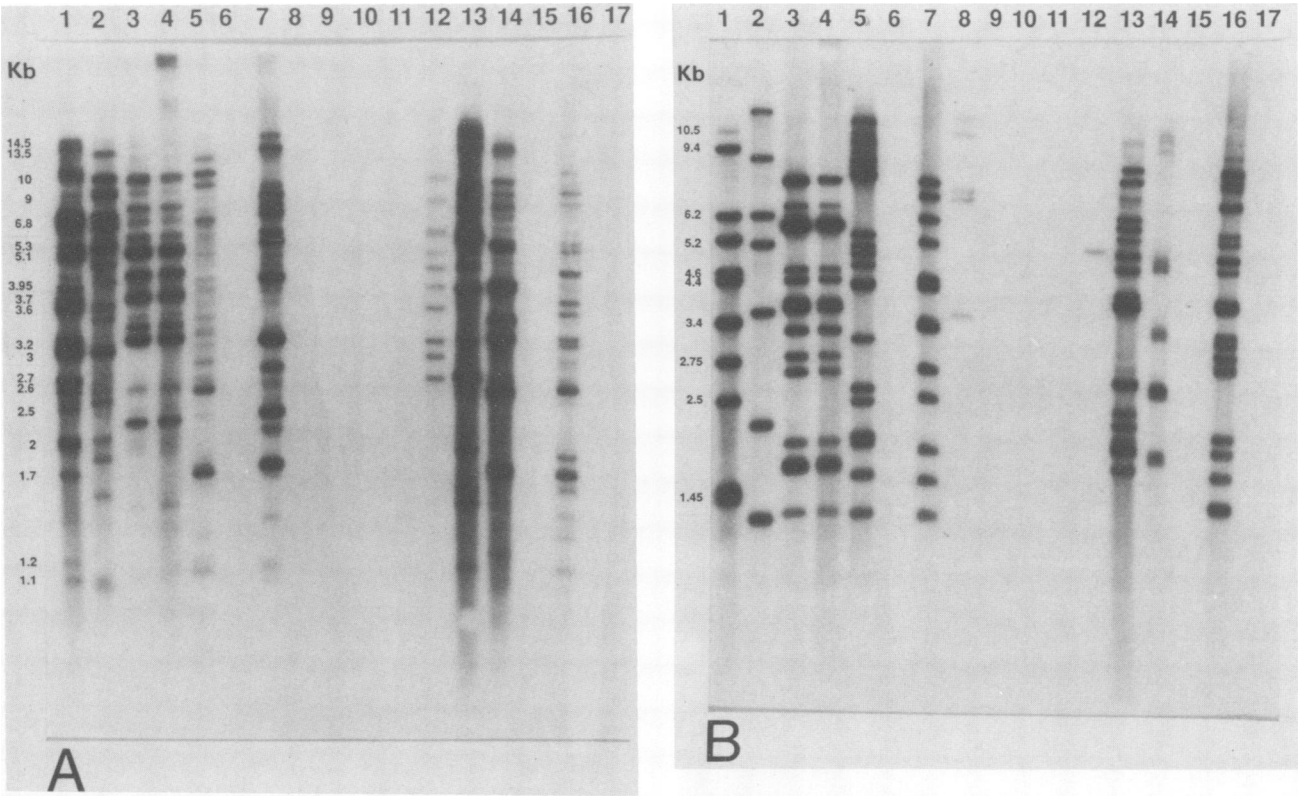

FIG. 2. Hybridization patterns of *Bgl*II-digested chromosomal *Leptospira* DNA probed with pL1 (A) and pL590 (B) under high-stringency conditions. Lanes: 1, australis; 2, bratislava; 3, hardjoprajitno (sample supplied by R. Farina, Pisa, Italy); 4, hardjoprajitno; 5, pomona; 6, patoc; 7, icterohaemorrhagiae; 8, tarassovi; 9, ballum; 10, javanica; 11, grippotyphosa; 12, shermani; 13, bataviae; 14, zanoni; 15, mini; 16, canicola; 17, saxkoebing. Molecular sizes were calculated for serovar australis on the basis of lambda DNA *Hin*dIII-digested marker.

The two probes hybridize extensively with the DNA of serovars australis, bratislava, pomona, icterohaemorrhagiae, bataviae, zanoni, and canicola and to a lesser degree with that of serovars shermani, grippotyphosa, and tarassovi. It should be noted that, while most of the serovars tested hybridize with both probes, grippotyphosa and tarassovi give a polymorphic pattern only with pL1 and pL590, respectively. No hybridization was detected for the serovars castellonis, javanica, mini, saxkoebing, and the nonpathogenic serovar patoc.

Under conditions of low stringency, it was also possible to detect a polymorphic pattern for the serovars castellonis and mini, as well as for tarassovi and grippotyphosa when they were probed with pL1 (Fig. 3A) and pL590 (Fig. 3B), respectively, while javanica gave a faint hybridization signal. Saxkoebing and patoc again did not hybridize.

Besides the different intensities of the autoradiographic signals, the size and the number of the fragments detected were unique for each serovar examined, allowing its identification by a specific polymorphic pattern. As a representative example, we analyzed the behavior of the serovars australis, bratislava, and lora, all belonging to the serogroup Australis. Genomic DNA was digested with the restriction enzymes *Bgl*II, *Pst*I, *Eco*RI, *Hin*dIII, and *Hae*III and hybridized to pL1 and pL590. Serovar australis was easily differentiated with both probes and with all the enzymes tested, while lora and bratislava gave very similar patterns with most enzymes tested and yielded different bands only when digested with *Bgl*II and *Eco*RI (Fig. 4).

A series of Southern blot experiments, using 12 probes present as one to four copies in the hardjoprajitno genome, confirmed the results obtained with pL1 and pL590: a high degree of homology was found between serovar hardjo genotype hardjoprajitno and serovars australis, bratislava, pomona, icterohaemorrhagiae, bataviae, zanoni, and canicola. A lower hybridization was observed with grippotyphosa, shermani, tarassovi, castellonis, javanica, and mini, while no signal was visible for saxkoebing and patoc (data not shown).

REA and Southern analysis within serovar hardjo. In order to test the ability of our probes to discriminate genotypes belonging to the same serovar, we hybridized *Bgl*II-digested hardjoprajitno and hardjobovis DNA with pL1, pL590, and the 12 probes mentioned above. Most probes (including pL1) resulted in faint or absent hybridization (data not shown) with the heterologous DNA from hardjobovis; nevertheless, pL590 hybridization yielded distinct polymorphic patterns and a better signal, which allowed us to discriminate the two genotypes. This analysis was applied to the identification of field strains 69 and 70, which were assigned to the genotype hardjobovis (Fig. 5).

Identification of the repetitive elements within clones pL1 and pL590. A restriction enzyme map of clone pL590 is presented in Fig. 6. Eight fragments were obtained from the digestion of clone pL590 with the restriction enzymes *Hin*dIII, *Xba*I, *Acc*I, *Eco*RI, *Pst*I, and *Hin*cII, alone or in combination. Each of these fragments, named a through h, was purified, labelled, and hybridized to *Bgl*II-digested DNA

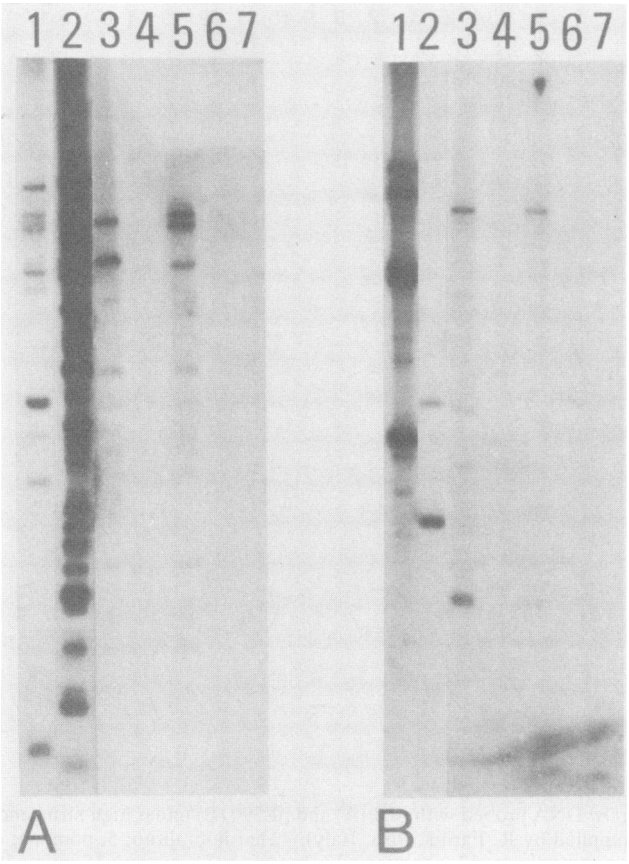

FIG. 3. Hybridization patterns of *Bgl*II-digested chromosomal *Leptospira* DNA probed with pL1 (A) and pL590 (B) under low-stringency conditions. Lanes: 1, tarassovi; 2, grippotyphosa; 3, castellonis; 4, javanica; 5, mini; 6, saxkoebing; 7, patoc.

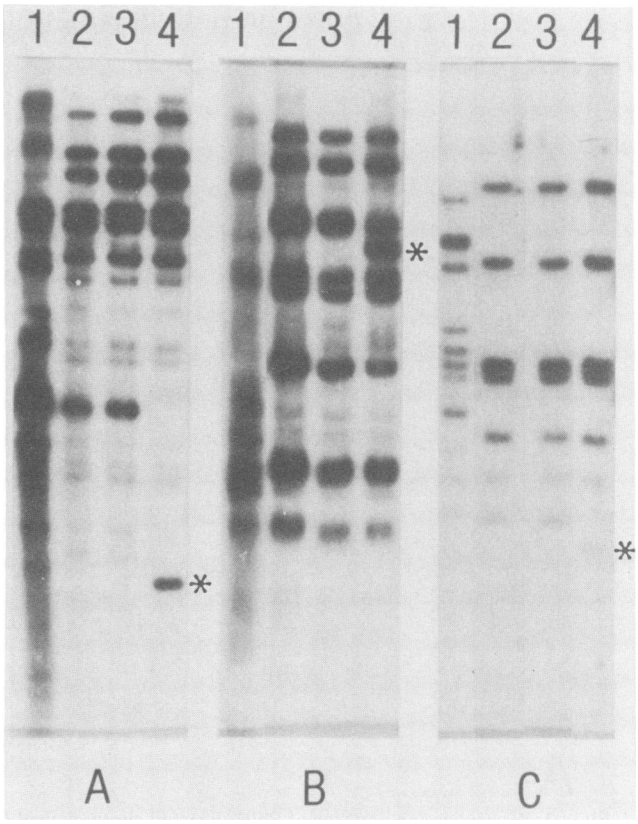

FIG. 4. Genomic DNAs of serovars australis (lanes 1), bratislava (strain Riccio 2) (lanes 2), bratislava (strain SV 396) (lanes 3), and lora (lanes 4) digested with *Bgl*II (A and B) and *Eco*RI (C) and probed with pL1 (A and C) and pL590 (B). The asterisks in lanes 4 indicate differences between lora and bratislava.

from hardjoprajitno, hardjobovis, and tarassovi (data not shown). The *Acc*I-*Eco*RI fragment e is the only one presenting a polymorphic pattern with the hardjobovis and tarassovi genomes, demonstrating that the repetitive element is present within this 619-bp fragment (Fig. 7). Comparison with the whole pL590 insert shows four extra bands (indicated by asterisks in Fig. 7) which are present in the lane containing hardjoprajitno DNA. They are due to a second independent repetitive element spanning fragments a and b. This element appears to be restricted to the "high homology group" serovars: hardjo genotype hardjoprajitno, australis, bratislava, pomona, icterohaemorrhagiae, bataviae, zanoni, and canicola.

A restriction enzyme map of pL1 was also constructed (Fig. 6), and unique restriction sites for *Pst*I, *Sma*I, and *Sal*I were located within the cloned insert. We followed a strategy identical to the one described above to map the repetitive element to fragment m. In this case, the hybridization patterns obtained with the 800-bp fragment m and with the whole pL1 insert are perfectly superimposable (Fig. 8).

DISCUSSION

Since we were interested in isolating clones containing repetitive sequences, we based our screening protocol on the relative intensity of the autoradiographic signal from individual colonies after hybridization with labelled genomic DNA. This approach led us to select 22 clones, some of which gave identical polymorphic hybridization patterns, as expected for random cloning of truly repetitive elements.

We conducted detailed hybridization studies with clones pL1 and pL590, which contain distinct repetitive elements, showing that they are able to identify the majority of the serovars tested. The patterns obtained were highly reproducible when several isolates of the same serovar were compared (10a).

While REA analysis is sufficient to characterize most *Leptospira* serovars, the use of Southern blot hybridization is particularly fitted to distinguish genetically similar ones (29). Furthermore, the polymorphic patterns obtained from repetitive sequences, shown in this and other works (25, 29), are made of a limited number of bands and are easier to interpret than REA (25).

The potential usefulness of our probes for practical purposes was assessed for some serovars of the serogroup Australis, which are widespread in Italy and whose genetic similarity makes discrimination by classical methods difficult. We have shown that pL1 and pL590 yield different polymorphic patterns with serovars australis, lora, and bratislava. Recently, Ellis et al. (3) have reported an REA study on serogroup Australis which also achieves serovar differentiation.

Another important aspect of *Leptospira* characterization relates to discrimination of isolates within the same serovar.

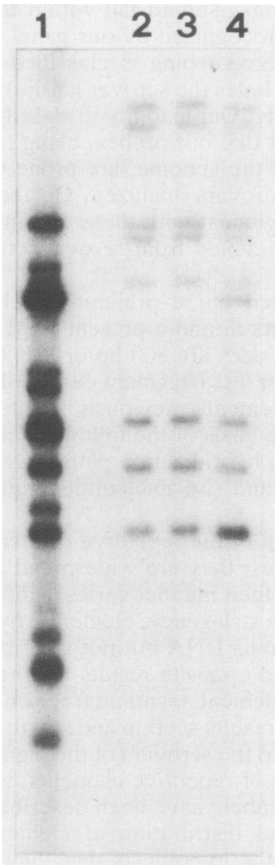

FIG. 5. Genomic DNA of the strain Hardjoprajitno (lane 1), field-isolated strain 69 (lane 2), field-isolated strain 70 (lane 3), and hardjobovis strain Sponselee (lane 4) digested with *Bgl*II enzyme and hybridized with pL590.

We performed Southern blot analysis of field strains 69 and 70, both assigned to the serovar hardjo on a serological basis. When compared with hardjoprajitno and hardjobovis strain Sponselee, the two field strains were clearly similar to

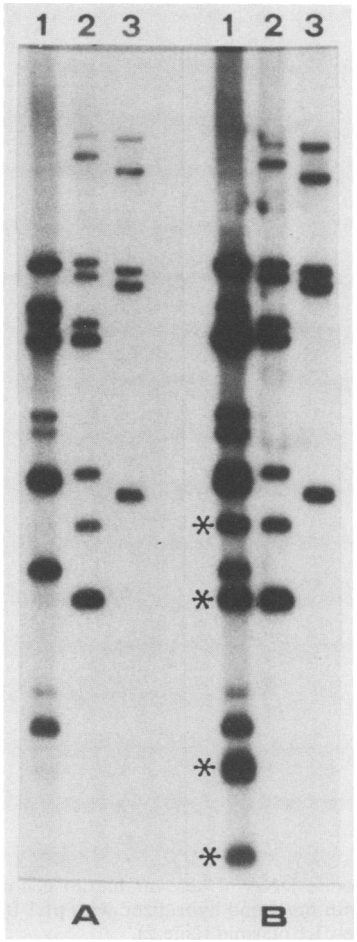

FIG. 7. *Bgl*II-digested genomic *Leptospira* DNAs of hardjoprajitno (lanes 1), hardjobovis (strain Sponselee) (lanes 2), and tarassovi (lanes 3) hybridized with pL590 fragment e (A) and the entire pL590 plasmid (B). The asterisks in lane 1, panel B indicate hybridization bands which are not due to fragment e.

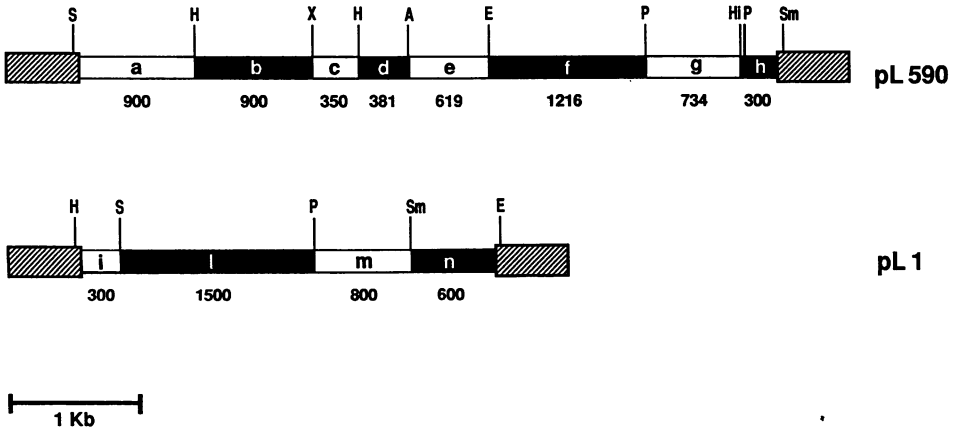

FIG. 6. Restriction enzyme map of pL590 and pL1. Plasmid sequences are represented by hatched boxes. E, *Eco*RI; P, *Pst*I; X, *Xba*I; H, *Hin*dIII; A, *Acc*I; Hi, *Hin*cII; S, *Sal*I; Sm, *Sma*I. The letters (a, b, c, etc.) indicate the fragments used as probes in Southern blot experiments to localize the positions of the repetitive elements. No cutting sites were found for the enzymes *Sma*I and *Sal*I in the pL590 insert or for *Eco*RI and *Hin*dIII in the pL1 insert.

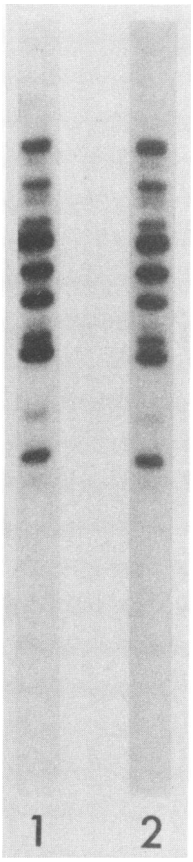

FIG. 8. Genomic DNA of serovar hardjo genotype hardjoprajitno digested with *Bgl*II and hybridized with pL1 fragment m (lane 1) and the entire pL1 plasmid (lane 2).

the latter and therefore belong to the genotype hardjobovis. The additional 6.5-kb hybridization band visible in strain Sponselee may be related to the genetic variability of the genotype (21).

The complete lack of hybridization to genomic DNA of bacteria commonly found as contaminants of *Leptospira* isolates indicates that pL1 and pL590 may be useful diagnostic tools, although more experiments will be required to establish the lack of cross-hybridization to a larger number of organisms.

Leptospira serovars have been classified into homology groups on the basis of their overall genomic sequence similarity, as assayed by Southern blot and dot blot experiments with total genomic DNA as a probe (8, 10, 19) or by the hydroxyapatite-DNA binding method (24).

The results presented here provide additional data on the degree of relatedness within our panel of serovars and are substantially in agreement with the studies mentioned above. Following the scheme proposed by Nielsen et al. (10), we assign pomona, icterohaemorrhagiae, lora, zanoni, canicola, bratislava, australis, and bataviae to a high homology group. All of these serovars have in common a strong hybridization signal when probed with our clones of hardjoprajitno genomic DNA. The low homology group includes tarassovi, castellonis, javanica, mini, hardjobovis, shermani, and grippotyphosa, and a third group comprises the nonpathogenic serovar patoc, which does not hybridize.

A notable exception is serovar saxkoebing, which, according to Yasuda et al., should fall within the high homology group but is not recognized by our panel of probes even at low stringency. Saxkoebing is classified in the serogroup Sejroe (which includes the serovar hardjo) on the basis of its antigenic reactivity. Our inability to classify saxkoebing may be due to the fact that our probes, being representative of a minor fraction of the genome, are prone to miss important features of the serovars analyzed. On the other hand, our results are in agreement with those obtained by Van Eys et al., using probes cloned from serovar icterohaemorrhagiae (25).

We have carried out a preliminary characterization of repetitive elements m and e present in pL1 and pL590 and shown that their sizes are 800 bp or less (fragment m from pL1) and 619 bp or less (fragment e from pL590). They differ from repetitive elements previously isolated from hardjobovis (27, 28) on the basis of the following criteria: (i) genome copy number, (ii) hybridization patterns with serovars analyzed, (iii) size, and (iv) nucleotide sequence comparison (10a).

The distribution of the repetitive elements presents some interesting features: they are widespread among the pathogenic strains, but their number varies in different serovars; in conditions of high stringency, element e hybridizes to tarassovi and hardjobovis DNA but not to grippotyphosa DNA, while we obtained opposite results for the hybridization of element m. The element spanning fragments a and b, briefly mentioned in the results section and still to be characterized, seems restricted to the serovars of the high homology group.

Several classes of repetitive elements, widely different in size and copy number, have been described for bacteria (4, 11). The size and distribution of elements e and m are compatible with the hypothesis that they are transposable elements or derive from ancient transposition events. This hypothesis would explain their presence in most, but not all, of the *Leptospira* serovars analyzed and their variable copy numbers; more work, especially at the DNA sequence level, is required to clarify this issue.

The cloning and sequencing of these elements from serovars other than hardjoprajitno will be facilitated by polymerase chain reaction-mediated amplification with specific primers. This technique is also being tested for its potential diagnostic application. A future direction of this work will be the cloning and characterization of additional repetitive elements from serovars belonging to our low homology group or from serovar patoc to provide a complete panel of diagnostic probes and possibly a better insight into the biology of repetitive sequences in the genus *Leptospira*.

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