JOURNAL OF CLINICAL MICROBIOLOGY, May 1991, p. 1042–1048
0095-1137/91/051042-07$02.00/0

Vol. 29, No. 5

Characterization of Serovars of the Genus *Leptospira* by DNA Hybridization with Hardjobovis and Icterohaemorrhagiae Recombinant Probes with Special Attention to Serogroup Sejroe

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Received 11 December 1990/Accepted 12 February 1991

Recombinant DNA probes derived from genomic libraries of serovars hardjobovis and icterohaemorrhagiae were applied for the characterization of leptospires. Differences in hybridization signals in combination with the banding pattern appear to provide good characteristics for strain typing. The banding patterns were easy to distinguish, since the recombinant DNA probes hybridized with a limited number of fragments. They were also indicative of genomic relationships between serovars. The probes suggested the existence of four subgroups with extensive genomic homology within the serogroup Sejroe. A number of serovars outside the serogroup Sejroe showed genomic homology with these subgroups. Amplification with the polymerase chain reaction showed a correlation with the genomic homologies demonstrated by Southern analysis. Knowledge about genomic relationships between leptospiral strains, as revealed by Southern analysis, may lead to a more rational approach for primer selection for polymerase chain reaction or cloning of particular genes.

Classification and identification of pathogenic leptospires rely upon the agglutination-absorption test. The reproducibility of this standard method is not satisfactory. The method is laborious and time-consuming. In 1967, Kmety proposed a system of antigenic factor analysis which used specifically absorbed sera for the taxonomy of leptospires (6). This method was a considerable improvement, but batch-to-batch variations between factor sera may lead to a lack of reproducibility (21, 22). Immunological techniques have become more rapid and specific since monoclonal antibodies have become available (7, 9, 18). In most cases, these methods have provided an acceptable degree of identification. However, a number of serovars cannot be identified by these methods. For instance, within the serogroup Sejroe, no differences could be demonstrated between serovars hardjobovis and hardjoprajitno. Another drawback of the serovar classification is the lack of any direct relation with clinical manifestation, geographical distribution, or other epidemiological markers.

As an alternative to serology, DNA-based techniques have been explored for the characterization of leptospiral strains. Two types of techniques have been tested: restriction endonuclease analysis (REA) and hybridization-annealing techniques. REA can be useful for differentiating between leptospiral serovars or even between strains (3, 4, 13, 16, 17, 21, 22). DNA hybridization with total DNA probes, as applied in dot blot, in situ, and hydroxylapatite assays, has shown that these techniques might be useful for identification and even classification of leptospires (19, 20, 25). Recombinant DNA probes facilitated the distinction between leptospiral strains, as was demonstrated for hardjobovis and hardjoprajitno (10, 11, 24). Southern analysis, combining the advantages of REA and hybridization techniques, will not only differentiate between strains but will also show homologies among them.

Recently, the polymerase chain reaction (PCR) has been tested as a system for detection (and identification) of leptospires. It appears to be a good technique for the early detection of leptospiral infections (23). Further application of the PCR will require the generation of common, as well as specific, primers. The synthesis of PCR primers demands a rational approach. The serovar classification system gives no guidelines as to from which serovars such primers should be derived. Serological and genomic typings do not appear to corroborate with each other (10, 14, 15, 24, 25). Therefore, it is necessary to obtain insight to the degrees of genomic homology between leptospiral strains. This knowledge will indicate the strains from which libraries have to be made and the strains with which such libraries have to be screened in order to obtain common or specific sequences from which PCR primers can be derived. Southern analysis is a method that will provide information about the presence of particular sequences and their arrangement in the genome.

The aim of this study was to investigate whether characterization by Southern analysis can be used for the genomic grouping of leptospiral serovars. Knowledge of the genomic organization of leptospires is important because the genomic relationships suggested by Southern analysis will provide a basis for primer selection in PCR, for probe selection in hybridization assays, and for cloning of pathologically important genes such as sphingomyelinase. All these techniques will help to improve detection and identification of leptospires.

MATERIALS AND METHODS

Leptospiral strains. Leptospiral serovars used in this investigation are listed in Table 1. Culturing conditions for these leptospires were essentially as described by Johnson and Harris (5).

Preparation of chromosomal DNA. Leptospiral cultures

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TABLE 1. Serovars used in this study

Species[a]	Serovar	Strain[b]	Origin	Serogroup
Alstonii	Bim	1051	Barbados	Autumnalis
	Grippotyphosa	Moskva V	USSR	Grippotyphosa
	Mozdok	5621	USSR	Pomona
Borgpet	Arborea	Arborea	Italy	Ballum
	Balcanica	1627 Burgas	Bulgaria	Sejroe
	Ballum	Mus 127	Denmark	Ballum
	Dikkeni	Mannuthi	India	Sejroe
	Guangdong	1853	China	Ballum
	Hardjobovis	Sponselee	Netherlands	Sejroe
	Istrica	Bratislava	Czechoslovakia	Sejroe
	Javanica	V Bat 46	Indonesia	Javanica
	Nyanza	Kibos	Kenya	Sejroe
	Polonica	493 Poland	Poland	Sejroe
	Saxkoebing	Mus 24	Denmark	Sejroe
	Sejroe	M84	Denmark	Sejroe
	Sorexjalna	Sorex Jalna	Czechoslovakia	Javanica
	Tarassovi	Perepelicin	USSR	Tarassovi
Interro	Australis	Ballico	Australia	Australis
	Autumnalis	Akiyami A	Japan	Autumnalis
	Bataviae	Swart	Indonesia	Bataviae
	Bratislava	Jez Brat	Czechoslovakia	Australis
	Canicola	H. UtrechtIV	Netherlands	Canicola
	Copenhageni	M20	Denmark	Icterohaemorrnagiae
	Geyaweera	Geyaweera	Sri Lanka	Sejroe
	Haemolitica	Marsh	Malaysia	Sejroe
	Hardjoprajitno	Hardjopra	Indonesia	Sejroe
	Hebdomadis	Hebdomadis	Japan	Hebdomadis
	Icterohaemorrhagiae	RGA	Germany	Icterohaemorrhagiae
	Lai	Lai	China	Icterohaemorrhagiae
	Medanensis	Hond HC	Indonesia	Sejroe
	Naam	Naam	Indonesia	Sejroe
	Pomona	Pomona	Australia	Pomona
	Ricardi	Richardson	Malaysia	Sejroe
	Romanica	LM 294	Rumania	Sejroe
	Schueffneri	V1.90C	Indonesia	Canicola
	Wolffi	3705	Indonesia	Sejroe
Noguchii	Fortbragg	Fort Bragg	USA	Autumnalis
	Panama	CZ 214	Panama	Panama
	Proechimus	1161 U	Panama	Pomona
Santaro	Bakeri	LT 79	USA	Tarassovi
	Borincana	HS 622	Puerto Rico	Hebdomadis
	Caribe	TRVL 61866	Trinidad	Sejroe
	Gorgas	1413 U	Panama	Sejroe
	Guaricura	Bov G	Brazil	Sejroe
	Recreo	380	Nicaragua	Sejroe
	Shermani	1342 K	Panama	Shermani
	Trinidad	TRVL 34056	Trinidad	Sejroe
	Tropica	CZ 299	Panama	Pomona

[a] Genotype was deduced from Southern analysis. With the exception of saxkoebing, all serovars tested belong to the same species as those proposed by Yasuda and coworkers (25) or Kaufmann (5a). Abbreviations: alstoni, *L. alstonii*; borgpet, *L. borgpetersenii*; interro, *L. interrogans*; noguchii, *L. noguchii*; santaro, *L. santarosai*.

[b] V Bat, Veldrat Batavia 46; Jez Brat, Jez Bratislava; Hardjopra, Hardjoprajitno.

were centrifuged at 12,000 × *g* for 30 min at 4°C. The pellets were washed twice in phosphate-buffered saline (pH 7.4), centrifuged, and resuspended in lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl [pH 7.4]). Sodium dodecyl sulfate (SDS) and pronase (Sigma) were added to final concentrations of 0.5% and 100 μg/ml, respectively, and incubated for 2 h at 60°C. Proteinase K (Sigma) was then added to a concentration of 20 μg/ml, and the protein digestion was continued for another 2 h at 37°C.

The DNA solutions were subjected to phenol and chloroform extraction twice and were precipitated with ethanol (12). After two washes in 70% ethanol the DNAs were dried and redissolved in ultrapure water.

Molecular cloning. Recombinant DNA clones were derived from genomic libraries of serovar hardjobovis (strain HB013; generously provided by A. Thiermann) and serovar icterohaemorrhagiae (strain RGA). Genomic fragments were cloned in pUC19 by standard procedures (12).

TABLE 2. Grouping of the members of the serogroup Sejroe according to homologies as deduced from hybridization reactivity and similarity of hybridization pattern by various techniques

Group	Serovar	Hybridization[a]				PCR[b]	Hydr apat[c]
		Total bovis	Recom bovis	Total icter	Recom icter		
I	Hardjobovis	+++	+++	−	−	+	
	Balcanica	+++	+++	−	−	+	Borg
	Nyanza	+++	+++	−	−	+	Borg
	Dikkeni	+++	+++	−	−	+	
II	Saxkoebing	++	++	+	+	+	Inter
	Polonica	++	++	+	+	+	Borg
	Sejroe	++	++	+	+	+	Borg
	Istrica	++	++	+	+	+	Borg
III	Caribe	+	+	+	+	−	Santa
	Trinidad	+	+	+	+	−	Santa
	Gorgas	+	+	+	+	−	Santa
	Guaricura	+	+	+	+	−	Santa
	Recreo	+	+	+	+	−	Santa
IV	Haemolytica	−	−	+++	+++	−	Inter
	Prajitno	−	−	+++	+++	−	Inter
	Medanensis	−	−	+++	+++	−	Inter
	Geyaweera	−	−	+++	+++	−	Inter
	Ricardi	−	−	+++	+++	−	Inter
	Romanica	−	−	+++	+++	−	Inter
	Wolffi	−	−	+++	+++	−	Inter

[a] Hybridization was determined by the outcome of dot blot assays and Southern blotting. −, No or a very weak homology; +, weak homology; ++, moderate homology (simple banding pattern); +++, extensive homology (complex banding pattern). Abbreviations: Total bovis, total DNA probe of hardjobovis; Recom bovis, recombinant DNA probes of hardjobovis; Total icter, total DNA probe of icterohaemorrhagiae; Recom icter, recombinant DNA probe of icterohaemorrhagiae.

[b] Results of the assay using primers derived from a hardjobovis clone. Since quantitation of the amplification is a problem, the outcome of the assay is given as positive (+) or negative (−).

[c] Hydr apat, Classification by hydroxylapatite hybridization selection (25). Borg, *L. borgpetersenii*; Inter, *L. interrogans*; Santa, *L. santarosai*.

The hardjobovis clones, isolated from genomic libraries, were selected on the basis of a strong hybridization signal and high specificity for hardjobovis compared with that for hardjoprajitno (24). Some of the hardjobovis clones obtained were subcloned for sequencing. Clones obtained from genomic icterohaemorrhagiae libraries were selected on the basis of a strong hybridization signal. Clones that were specific for icterohaemorrhagiae or common for several serovars were selected by differential screening.

Southern analysis. For Southern analysis, chromosomal DNAs were digested for 4 h at 37°C with endonuclease *Hin*dIII or *Eco*RI by using 5 U of DNA per μg. The fragments were separated on 0.7% agarose gels, after which these gels were prepared for blotting by standard procedures (12). After blotting, filters were rinsed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and subsequently baked for 2 h at 80°C. Prehybridization and hybridization were performed in a solution containing 7% SDS, 1% bovine serum albumin, 2 mM EDTA, 0.5 mg of salmon sperm DNA per ml, and 0.5 M Na_2HPO_4–H_3PO_4 (pH 7.0) at a temperature of 68°C. Probe DNA was labeled by random priming to a specific activity of at least 10^8 cpm per μg of DNA, and 10^7 cpm was added to the hybridization solution. After hybridization, the filters were washed first in 3× SSC, then in 1× SSC–0.1% SDS, and finally in 0.1× SSC–0.1% SDS for 30 min each at 68°C. Autoradiography was performed at −70°C on Kodak film by using intensifying screens.

DNA amplification reaction. The PCR was performed in a Biomed thermocycler. *Taq* polymerase was purchased from Cetus-Perkin Elmer. In the assays used for this study, 10 ng of purified genomic DNA was added to the reaction mixture. The annealing temperature was 55°C. The procedure and chemicals used were as described previously (23).

RESULTS

At present, the most frequently used system for characterization and classification of leptospires is serogroup and serovar typing. This classification system uses the concept of a single pathogenic species, *Leptospira interrogans*. A few years ago Yasuda and coworkers (25) proposed a system of classification based on DNA hybridization. Although this classification system is not as commonly used nor as widely accepted as serotyping, it is used in this paper. This is done because our data support the phylogenetic concept of a genus *Leptospira* which contains many species (for this paper, *L. alstonii*, *L. borgpetersenii*, *L. interrogans*, *L. noguchii*, and *L. santarosai*). In Table 1, serovars are presented according to species, with additional information about the serotyping by conventional methods.

Seventeen recombinant DNA probes (together covering about 3% of the leptospiral genome) were tested by Southern analysis to examine the possibilities for characterization of leptospiral strains. The probes, selected by differential screening, hybridized efficiently with the homologous serovars. With nonhomologous serovars, the hybridization efficiency was in accordance with the multispecies classification but not with the serotyping classification for probes from both hardjobovis and icterohaemorrhagiae libraries. Within a serogroup, as demonstrated for serovars of the serogroup Sejroe, different levels and patterns of hybridization were observed (Table 2). For example, total hardjobovis DNA

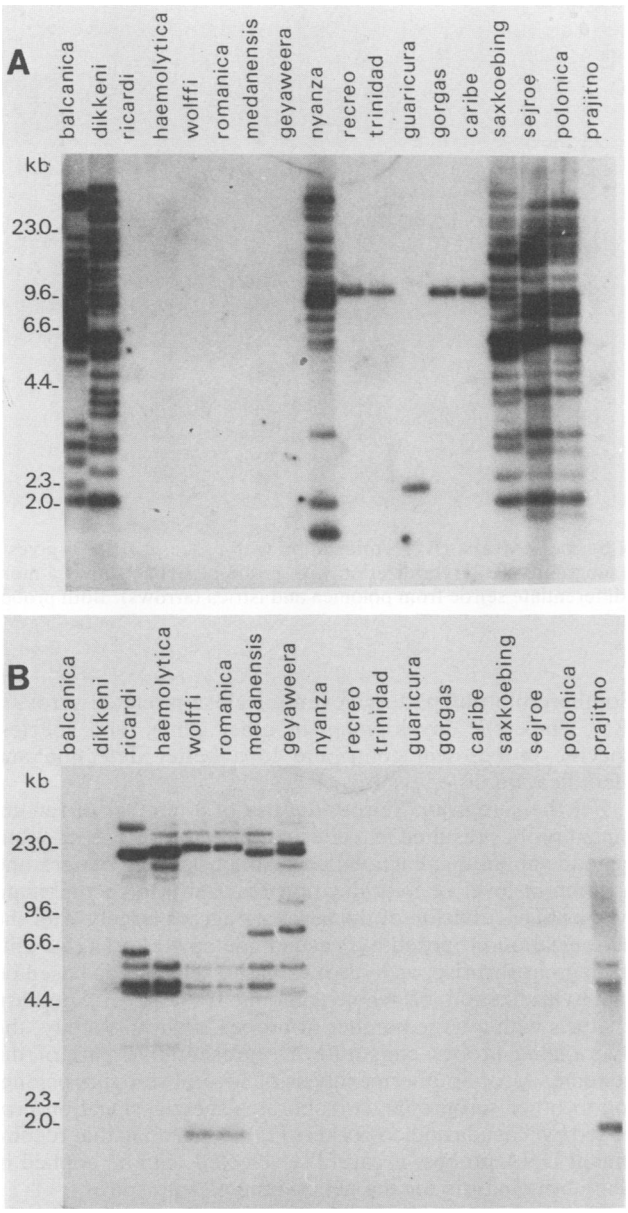

FIG. 1. Southern blots of gels with *Hin*dIII-digested genomic DNAs of Sejroe serovars. Variations in hybridization patterns within the Sejroe serogroup are demonstrated by probes pLBMb06 (derived from a hardjobovis library) (A) and pLIHp61 (derived from an icterohaemorrhagiae library) (B).

probes (used in a dot blot system) and recombinant probes from a hardjobovis library reacted strongly with serovars such as dikkeni and balcanica but not at all with serovars such as wolffi and haemolytica (Fig. 1) (Table 2). On the other hand, total DNA probes and recombinant probes from an icterohaemorrhagiae library hybridized strongly with serovars such as wolffi and haemolytica but not with serovars such as dikkeni and balcanica (Fig. 1) (Table 2). No hybridization with any of the probes was found for nonpathogenic strains such as patoc and illini (data not shown). In addition, results obtained for serovars belonging to other serogroups, such as Autumnalis, Hebdomadis, and Pomona, show no relation between serotyping and Southern analysis (Table 1).

For the serogroup Sejroe, recombinant probes from hardjobovis and icterohaemorrhagiae libraries showed (for 15 of 17 probes) a division, determined by the banding pattern and hybridization efficiency in Southern analysis, which was in agreement with the multispecies classification (Fig. 2A and 3A). A few probes gave specific banding patterns for almost all different serovars (Fig. 2B). Serovars istrica and polonica were identical for all probes tested, as were wolffi and romanica. Nevertheless, the serovar-specific probes indicated that serogroup Sejroe was divided into four genomic groups. For the serogroup Sejroe the following division was deduced.

Group I showed very strong hybridization and often showed complex banding patterns for hardjobovis probes but did not hybridize with icterohaemorrhagiae probes. The serovars hardjobovis, balcanica, nyanza, and dikkeni belong to this group. Serovars balcanica and nyanza belong to the species *L. borgpetersenii*. Serovars dikkeni and hardjobovis display the same patterns and should therefore be considered to belong to the same species.

Group II showed a moderate level of hybridization with the hardjobovis probes and a very weak hybridization with the icterohaemorrhagiae probes. This group comprised saxkoebing, sejroe, polonica, and istrica. All strains of this group came from Europe and belong to the species *L. borgpetersenii*, with the exception of serovar saxkoebing, which has been described previously as belonging to *L. interrogans* (25).

Group III hybridized weakly with the hardjobovis probes, as well as with icterohaemorrhagiae probes; often only one band was observed on the autoradiograms. Serovars caribe, gorgas, trinidad, recreo, and guaricura made up this group. All strains in this group were isolated in Latin America and belong to the species *L. santarosai*.

Group IV showed no hybridization with hardjobovis probes, while a strong signal was observed when hybridized with icterohaemorrhaghiae probes. Serovars haemolytica, hardjoprajitno, medanensis, geyaweera, ricardi, romanica, and wolffi belonged to this group. With the exception of romanica, all strains of this group are from Southeast Asia and belong to the species *L. interrogans*.

Serovars belonging to other serogroups displayed banding patterns and hybridization efficiencies similar to one of the subgroups of serogroup Sejroe and could accordingly be assigned to one of the species (Table 1) (Fig. 3A).

From the sequence of clone pLBEc23s (hardjobovis library), primers have been derived for PCR. By Southern analysis under high-stringency conditions the presence of similar sequences was demonstrated in more than 40 serovars within and outside the serogroup Sejroe (a selection is shown in Fig. 3A). This probe also divided the serogroup Sejroe into the same four (sub)groups. Only serovars that have a strongly hybridizing 6-kb band on Southern blots after *Eco*RI digestion showed an amplification in the PCR (Fig. 3B). Within the serogroup Sejroe these are the serovars of subgroup I and II. In addition, serovars of other serogroups, such as arborea and guangdong (Fig. 3) and sorexjalna and perepelicin (data not shown), give a positive PCR. Serovars within the serogroup Sejroe such as recreo and trinidad (which showed a weak band of different size) gave no amplification in the PCR. So as far as was tested, only serovars belonging to *L. borgpetersenii*, as defined by Yasuda et al. (25), showed amplification in the PCR with these primers.

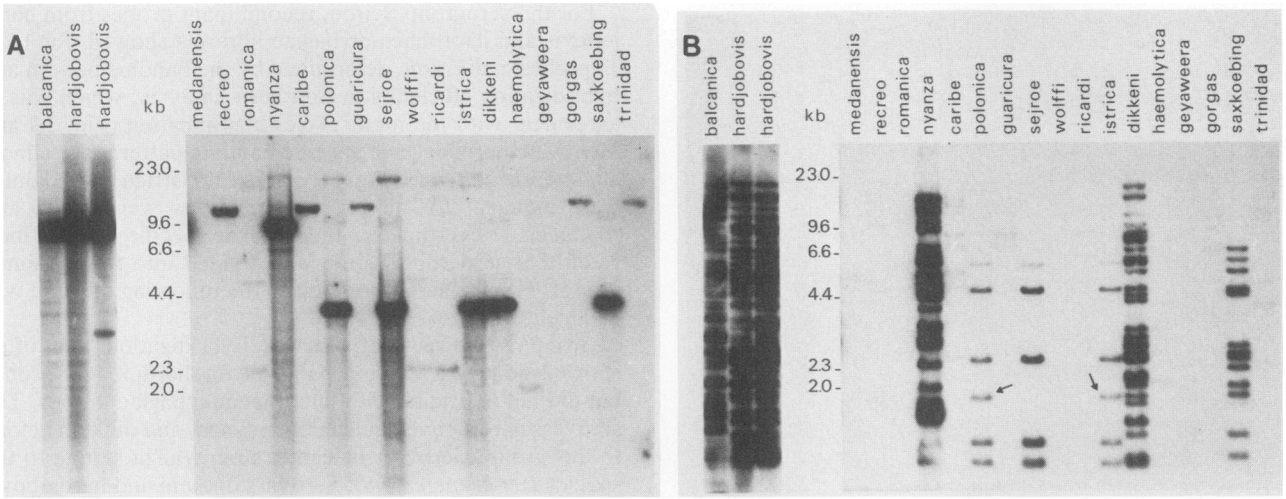

FIG. 2. Southern blots of gels with *Hin*dIII-digested genomic DNAs of Sejroe serovars. (A) Hybridization with probe pLBEc07s gives a simple hybridization pattern that divides the serogroup into four distinct subgroups. (B) Hybridization with probe pLBHi26 shows a more complex pattern but the same division. With this probe it was possible to differentiate sejroe from polonica and istrica (arrows). Both probes were derived from hardjobovis libraries.

DISCUSSION

A DNA-based method such as REA shows differences between genomic DNAs by restriction fragment patterns. The level of annealing between total DNA probes and target DNA in in situ, dot blot, and hydroxylapatite hybridizations reflects degrees of homology between genomic DNAs. Southern analysis combines these two parameters. The results presented suggest that Southern analysis with recombinant DNA probes is a reliable technique for the characterization of leptospiral strains. Since recombinant DNA probes react with a limited number of restriction fragments, the banding pattern is simplified and interpretation is facilitated. This gives an advantage over REA, a technique that provides a complex banding pattern that even with computer assistance cannot always be completely analyzed (4). In a few cases, Southern analysis can distinguish strains that show identical patterns in REA, as was shown for polonica and istrica versus sejroe.

Information about homology between two strains can be obtained from annealing experiments between genomic DNAs, as is done with dot blot, in situ, or hydroxylapatite hybridizations (19, 20, 25). However, each of these methods provides information only on the overall homology of the genome of the strains. Southern analysis utilizes recombinant DNA probes which are better defined and more specific than total DNA probes (10, 15, 24, 26). Southern blots reveal a banding pattern, in addition to the degree of hybridization reactivity. Thus, Southern blotting simultaneously provides information about the homology and the genomic arrangement of a particular DNA sequence from different leptospiral strains. Previously, this approach has been tested with rRNA probes (1, 2, 15). Such probes are certainly valuable, since they are likely to hybridize to all leptospiral serovars. For detection of infections this is an advantage. Point mutations within the genes and variations between the two gene clusters (2) can be used for identification by Southern analysis (2, 15). However, rRNAs are rather strictly conserved, and cross-hybridization with other microorganisms cannot be excluded. In addition, the presence of two separate rRNA gene clusters may result in a great variety of Southern blotting patterns even for closely related serovars (15). Thus, the development of other genus- and species-specific probes will contribute to a better detection and identification of leptospires.

For the serogroup Sejroe, the use of a number of recombinant probes resulted in a division of the serogroup into four genetic subgroups, each with a distinct banding pattern and a different level of hybridization reactivity to recombinant probes. This division of the serovars agrees largely with the classification proposed by Yasuda and coworkers (25). This is no great surprise, as hydroxylapatite selection is based on the hybridization of whole genomic DNAs. In Southern analysis with a large number of probes, as in this study, the hybridizing probes constitute a representative part of the genome. Also, Southern analysis of several serovars belonging to other serogroups corroborates the classification proposed by Yasuda and coworkers (25), confirming that recombinant DNA probes, if carefully selected, can be applied in Southern analysis for the genotyping of leptospires.

The division of serogroup Sejroe made on the basis of Southern analysis revealed a remarkable correlation with the geographical origins of the strains. Southern analyses of serovars belonging to other serogroups and of data from other investigators (5a, 14, 15, 25) support the idea of a relation between genotype and geographical origin of a strain. Strains belonging to the species *L. santarosai* and *L. noguchii* are all found in the "New World," whereas strains in the "Old World" belong to species *L. interrogans* (mostly Asia) and *L. borgpetersenii* (mostly Europe).

A greater genomic homology between two strains results in a greater parity of hybridization levels and a greater similarity of the banding patterns. Such information on the composition of the genome of leptospiral strains will allow a more efficient use of the PCR. Southern blots show the presence of a selected sequence of DNA, its copy number, and its genomic arrangement in a particular strain. Our data show that the mere presence of a sequence as deduced from hybridization assays is no guarantee of an amplification by a PCR. The position or arrangement of a particular sequence in the genome might be of equal importance. Thus, as

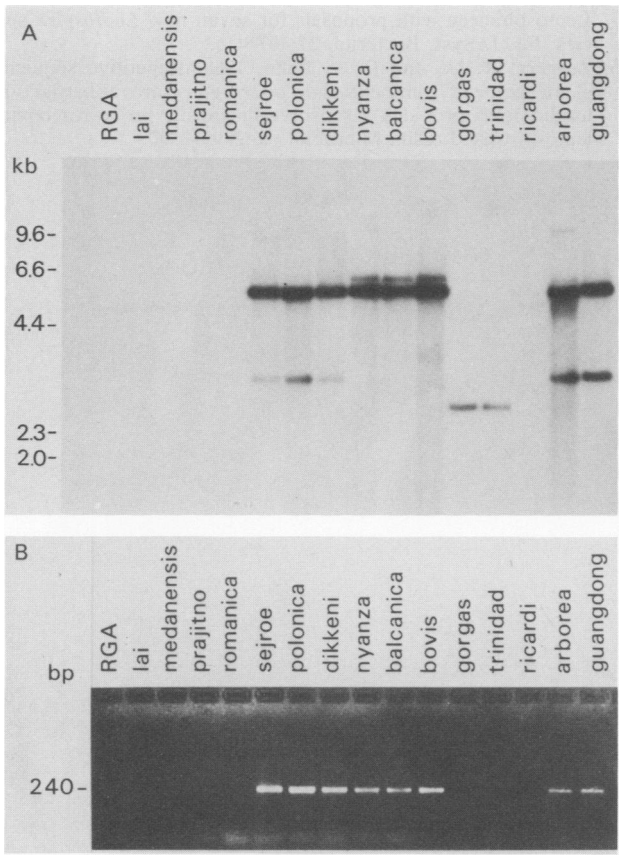

FIG. 3. (A) Southern blot of a gel with *Eco*RI-digested genomic DNAs of serovars within and outside the serogroup Sejroe. The blot was hybridized with the probe pLBEc23s (hardjobovis library). From this clone PCR primers were derived. (B) PCR-amplified samples were analyzed by electrophoresis on a 1.7% agarose gel.

powerful new techniques such as PCR set new standards for detection (and identification) of leptospires, it will be necessary to have detailed genomic information, such as that provided by Southern blots.

This study shows that Southern analysis can characterize leptospires on the basis of the hybridization banding pattern with recombinant DNA probes. It can also provide information about genomic relationships between strains. Such information will facilitate the application of powerful DNA-based diagnostic techniques, such as PCR, for leptospiral infections.

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