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Relationship among Selected *Leptospira interrogans* Serogroups as Determined by Nucleic Acid Hybridization†

JUDITH N. NIELSEN,[1]* CHARLES H. ARMSTRONG,[1] AND NIELS C. NIELSEN[2]

Department of Veterinary Pathobiology and Animal Disease Diagnostic Laboratory, School of Veterinary Medicine,[1] and U.S. Department of Agriculture Agricultural Research Service, Department of Agronomy,[2] Purdue University, West Lafayette, Indiana 47907

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Leptospiral DNAs from a variety of *Leptospira interrogans* serogroups of veterinary significance, as well as a nonpathogenic leptospira, were compared by Southern blot hybridization of *Eco*RI-digested genomic DNA. The serogroups examined could be assigned to one of three groups on the basis of the degree of cross-hybridization between genomic DNAs. Only a few restriction fragments hybridized between the three groups, and most of these were shown to contain ribosomal DNA. The restriction fragment length polymorphism observed among the intergroup hybridizations allowed differentiation among serogroups and, in some cases, serovars. Under the hybridization conditions used, no hybridization was observed between leptospiral DNA and *Leptonema*, *Escherichia coli*, or porcine DNA.

The genus *Leptospira* is composed of three species: *Leptospira interrogans* (parasitic or pathogenic organisms), *Leptospira biflexa* (free-living saprophytic bacteria), and *Leptospira parva* (an apparently nonpathogenic form isolated from tap water) (6, 7). The last species is distinguished from the other two species on the basis of biochemical characteristics that are intermediate between those of *L. interrogans* and *L. biflexa*. *Leptospira illini*, an organism isolated from the urine of a bull, was originally included in the genus *Leptospira*. However, because it has different structural and biochemical properties from other *Leptospira*, a new genus *Leptonema* has been proposed (5).

The species *L. interrogans* contains more than 170 serovars that are organized into 19 serogroups on the basis of antigenic relatedness (7). Conventional serogroup and serovar identification of leptospira is based on microscopic agglutination tests and cross-absorption with group- and serovar-specific hyperimmune serum (3). However, recent studies in which restriction endonuclease analysis was used have demonstrated differences within serogroups that are not detected by conventional microscopic agglutination tests (11, 12, 14). As a consequence, restriction endonuclease analysis is beginning to be used for identification of leptospiral isolates.

Attempts have been made to classify *Leptospira* on the basis of the degree of homology between genomic DNA from different *Leptospira* species and serogroups. These studies have shown that there are differences in the degree of homology between members of the genus that are detectable by DNA cross-hybridization and by the guanine-plus-cytosine (G+C) content of the genomic DNA (1, 4, 9, 16).

The purpose of this study was to explore the degree of DNA relatedness among different leptospiral species and, in particular, among various *L. interrogans* type strains that represent serogroups of veterinary significance in the United States. Conditions are described that permit leptospiral serogroups to be distinguished from one another and from *Escherichia coli*, *Leptonema*, and porcine DNA by using genomic Southern blot hybridization.

MATERIALS AND METHODS

Leptospiral strains. Reference strains of *L. (Leptonema) illini* serovar illini (strain 3055), *Leptospira biflexa* serovar biflexa (strain codice), and *Leptospira interrogans* serogroup Australis serovars australis (strain Ballico), lora (strain Lora), muenchen (strain Muenchen L90), jalna (strain Jalna), and bratislava (strain Jez Bratislava) were kindly supplied by David Miller, National Veterinary Services Laboratory, Ames, Iowa. *L. interrogans* serogroup Sejroe serovar hardjo (strain Hardjoprajitno), serogroup Grippotyphosa serovar grippotyphosa (strain Andaman), serogroup Icterohaemorrhagiae serovar copenhageni (strain M 20), serogroup Ballum serovar ballum (strain Mus 127), serogroup Tarassovi serovar tarassovi (strain Perepelicyn), serogroup Canicola serovar canicola (strain Hond Utrecht IV), serogroup Pomona serovar pomona (strains pomona and kennewicki), and serogroup Autumnalis serovar autumnalis (strain Akiyami A) were obtained from the National Animal Disease Center, Ames, Iowa, and were maintained as type antigens for serologic testing at the Indiana Animal Disease Diagnostic Laboratory for the past 12 to 14 years. *L. interrogans* serogroup Sejroe serovar hardjo (strain hardjobovis) was obtained from Carole Bolin, National Animal Disease Center. Leptospira designated P3389-1 and 2 were field isolates from swine confirmed by the National Animal Disease Center to be *L. interrogans* serovar grippotyphosa by restriction endonuclease analysis. *E. coli* ribosomal DNA probes (pNO1300 containing the entire ribosomal DNA (genes coding for rRNA; rDNA) operon and pNO1311 containing the 16S rDNA gene) were kindly supplied by Masayasu Normura, University of California, Irvine.

Preparation of whole-cell DNA. The leptospira were grown in Ellinghausen-McCullough (Polysorbate 80) medium (3) to a nephlometry value of approximately 40 in 500-ml cultures. The cells were harvested by centrifugation, washed 3× in 0.01 M phosphate-buffered saline, pH 7.5, and stored at −80°C until used. Cellular DNA was extracted following lysis in the presence of lysozyme, proteinase K, and Sarkosyl; it was then purified by overnight cesium chloride cen-

* Corresponding author.

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trifugation as described by Thiermann et al. (14). The DNA was concentrated by ethanol precipitation and quantitated by spectrophotometry at 260 μm by the method of Maniatis et al. (10). Porcine DNA from neonatal swine liver was prepared and purified by using the same procedures as those for leptospiral DNA. *E. coli* DNA was purchased from Sigma Chemical Co., St. Louis, Mo.

Restriction endonuclease digestion and Southern hybridization of DNA. Leptospiral DNA (2-μg) samples were digested with *Eco*RI restriction endonuclease purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used according to the specifications of the manufacturer. Digested DNA fragments were separated by electrophoresis overnight in a 15 by 25 cm 0.7% agarose gel containing 150 μg of ethidium bromide. The gels were exposed to UV irradiation by transillumination and were photographed by using a red filter. The genomic DNA fragments were blotted onto Gene Screen Plus nylon membranes according to the recommendations of the manufacturer (Dupont, NEN Research Products, Boston, Mass.). Genomic DNA probes were prepared by nick translation of 2 to 9 μg of *Eco*RI-digested leptospiral DNA. Nick translation was performed in the presence of [α^{32}P]CTP according to the instructions of the manufacturer by using nick translation kits purchased from Bethesda Research Laboratories, Inc. The ribosomal DNA probes used were contained in plasmids pNO1300 and pNO1311 (2). These two clones contained the entire *E. coli* rDNA operon and the 16S ribosomal gene, respectively. Hybridization of the probes to the genomic Southern blots was carried out overnight at 42°C in 50% formamide (10). After hybridization, the filters were washed at room temperature for 1 h in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate. This wash was followed by two 1-h washes at 65°C in 0.1× SSC containing 0.1% sodium dodecyl sulfate. Autoradiographs were exposed at −80°C for 2 to 7 days.

RESULTS

Restriction endonuclease digests of genomic leptospiral DNA give complex patterns. Figure 1 shows typical *Eco*RI restriction digests of a number of the serovars included in this study. While differences can be detected between the serovars, the pattern complexity requires careful analysis. Any contaminating DNA from other sources would obscure the differences.

In an attempt to simplify the restriction fragment patterns and study the degree of relatedness among leptospiral serovars, genomic Southern blot hybridization was performed by using ^{32}P-labeled *Eco*RI-digested genomic DNA from various leptospiral serovars as a probe. Figure 2A illustrates a typical hybridization pattern obtained when genomic DNA from *L. biflexa* was used to probe blots of leptospiral genomic DNA that were similar to those illustrated in Fig. 1. There was extensive hybridization between the biflexa probe and biflexa DNA. However, very few *Eco*RI fragments from *L. interrogans* serovars hybridized with the biflexa DNA probe, and no discernable hybridization to fragments from *Leptonema illini* were evident. Some fragments from the *L. interrogans* serovars, such as the fragment of approximately 0.7 kilobase (kb), appeared to be the same size in all serovars. In other cases, the fragments that hybridized were characteristic of individual serovars. Examples of this include the 1.5-kb fragment of ballum or the 5- and 6-kb fragments of autumnalis. The average DNA restriction fragment sizes of these and other serovars that exhibited limited homology with either genomic biflexa, bratislava, or tarassovi DNA were determined from a number of Southern blots and are summarized in Table 1.

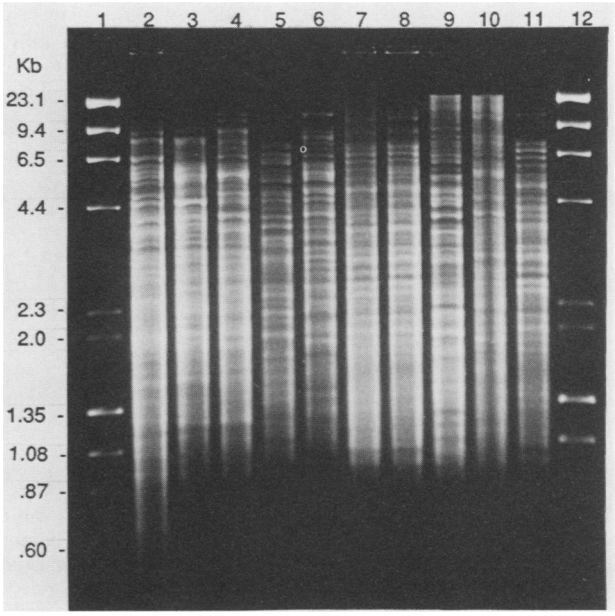

FIG. 1. *Eco*RI-digested genomic DNA fragments from various *L. interrogans* serovars separated by agarose–ethidium bromide gel electrophoresis. Lanes: 1 and 12, marker DNA of known fragment size; 2, bratislava; 3, australis; 4, autumnalis; 5, ballum; 6, pomona; 7, grippotyphosa field isolate P3389-2; 8, grippotyphosa; 9, tarassovi; 10, canicola; 11, grippotyphosa field isolate P3389-1.

Figure 2B shows that tarassovi genomic DNA probes hybridized extensively with both tarassovi and ballum in genomic Southern blots. The hybridization of restriction fragments of hardjoprajitno and australis was less extensive, and only a few fragments of other *L. interrogans* serovars hybridized with either ballum or tarassovi genomic DNA. These results were observed when probes of very high specific activity were used (approximately 2×10^{10} cpm/μg of DNA). When DNA probes of more moderate specific activity (approximately 2×10^{7} cpm/μg of DNA) were used, the restriction fragment pattern with hardjoprajitno and australis was less complex. In all cases, there was no detectable hybridization to genomic DNA from *E. coli* and *L. biflexa*.

Figure 2C shows that probes composed of bratislava genomic DNA hybridized extensively with bratislava and many of the other *L. interrogans* serovars but not with ballum and tarassovi DNA. In the case of the latter two serovars, only a few *Eco*RI fragments hybridized with the bratislava DNA probe. When bratislava genomic DNA was used to probe Southern blots of genomic DNA from serovars belonging to the Sejroe serogroup, a mixed pattern of hybridization emerged. There were extensive hybridization with hardjoprajitno, while only a few restriction fragments hybridized with hardjobovis genomic DNA. In similar experiments in which genomic DNA from ballum or tarassovi was used, there was extensive hybridization with hardjobovis but only a few hardjoprajitno restriction fragments hybridized.

To characterize the highly conserved DNA within the leptospiral genome that was revealed by our cross-hybridization experiments, rDNA probes were used to determine

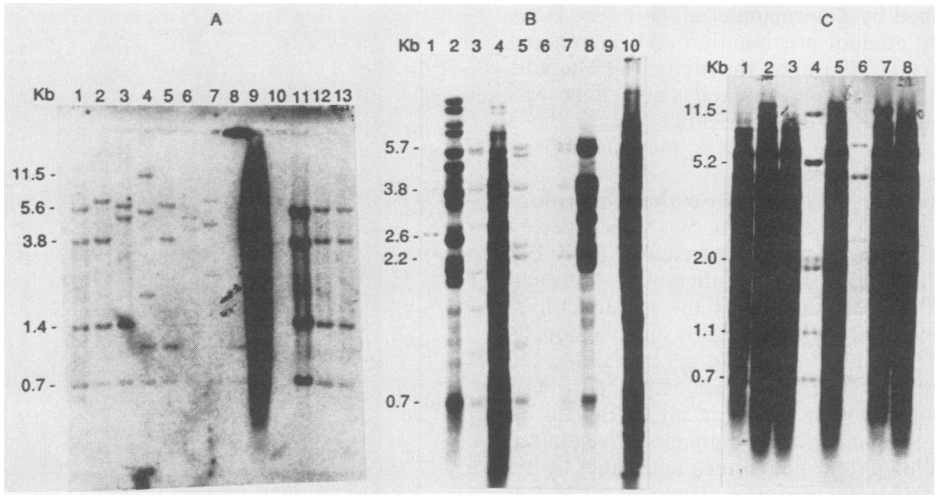

FIG. 2. (A) *Leptospira* serovar biflexa genomic [^{32}P]DNA was used to probe a Southern blot of *Eco*RI-digested leptospiral genomic DNA. Lanes: 1, bratislava; 2, australis; 3, autumnalis; 4, ballum; 5, pomona; 6, grippotyphosa; 7, tarassovi; 8, canicola; 9, biflexa; 10, illini; 11, jalna; 12, lora; 13, muenchen. (B) *Leptospira* serovar tarassovi genomic [^{32}P]DNA was used to probe a similar Southern blot. Lanes: 1, icterohaemorrhagiae; 2, hardjoprajitno; 3, bratislava; 4, ballum; 5, canicola; 6, biflexa; 7, pomona; 8, australis; 9, *E. coli;* 10, tarassovi. (C) *Leptospira* serovar bratislava genomic [^{32}P]DNA was used to probe a similar Southern blot. Lanes: 1, icterohaemorrhagiae; 2, hardjoprajitno; 3, bratislava; 4, ballum; 5, canicola; 6, tarassovi; 7, pomona; 8, australis.

if any of the conserved leptospiral *Eco*RI restriction fragments contained rDNA. The DNA inserts in pNO1300 and pNO1311, which contained the entire *E. coli* rDNA operon and the 16S *E. coli* ribosomal gene, respectively, were used to probe the leptospiral DNA. Figure 3A shows that each leptospiral serovar tested had three or four rDNA-containing *Eco*RI fragments that hybridized with the pNO1300 probe. These corresponded in size with most of the fragments observed when relatively nonhomologous leptospiral genomic DNA was used to probe Southern blots. The fragments found to contain rDNA are identified in boldface type in Table 1. Some restriction fragment length polymorphism was observed in the rDNA-containing DNA fragments. For example, biflexa could be identified by a unique 15.8-kb rDNA fragment. Tarassovi alone contained a 4.45-kb rDNA fragment. Only australis had a 1.72-kb rDNA fragment. While some restriction fragments did not differ significantly in size from one leptospiral serovar to another (for example, those of approximately 2.5, 2.0, 1.4, 1.1, and 0.7 kb), not all similarly sized fragments contained rDNA (Table 1).

Figure 3B illustrates a typical Southern blot in which pNO1311, which encoded only 16S rDNA, was used as probe. As anticipated, fewer restriction fragments hybridized with pNO1311 than with pNO1300. These fragments are identified by italicized boldface type in Table 1. The sizes of the restriction fragments that contained 16S rDNA varied from 4.45 to 1.1 kb. Tarassovi and ballum each had two fragments that hybridized with pNO1311, although the fragments were of different sizes (4.45 and 2.45 kb for tarassovi and 2.01 and 1.1 kb in the case of ballum).

DISCUSSION

Together, the data generated by using different leptospiral genomic DNA probes allowed us to sort the leptospiral strains included in this study into three distinct groups on the basis of the degree of cross-hybridization between their genomic DNAs. Leptospiral genomes within each group exhibited extensive cross-hybridization, whereas only a few DNA restriction fragments hybridized with genomic leptospiral DNA from other groups. Group 1 consisted of the nonpathogenic *L. biflexa* serovar biflexa (type codice); group 2 contained type cultures of *L. interrogans,* serogroups Tarassovi and Ballum; and group 3 contained the representatives of *L. interrogans* serogroups Australis, Autumnalis, Pomona, Grippotyphosa, Icterohaemorrhagiae, and Canicola. One exception to the above serogroup assignments emerged when the serogroup Sejroe was studied. Hardjobovis exhibited extensive homology with group 2 organisms, while hardjoprajitno exhibited extensive homology with group 3 organisms. Hence, we assigned *L. interrogans* serogroup Sejroe serovar hardjo (strain hardjobovis) to group 2 and serogroup Sejroe serovar hardjo (strain hardjoprajitno) to group 3.

An early study using thermal elution of leptospiral DNA separated the leptospira into four groups on the basis of duplexes trapped in agar (4). The pathogenic *L. interrogans* strains were assigned to two groups, and the nonpathogenic strains were also placed in two groups. Although only a limited comparison can be made between the conclusions drawn from that study and ours, the few strains both studies had in common were in agreement with regard to grouping on the basis of the extent of DNA homology.

In an expanded study that relied on DNA-DNA hybridization on membranes, Brendle et al. (1) recognized seven genetic groups, three "complex" classes of leptospira, with each class further subdivided into several groups on the basis of the extent of DNA homology. One of the three major complexes contained the pathogenic *L. interrogans* species and was divided into groups of organisms represented by bataviae, javanica, and ranarum. The bataviae group corresponded to our group 3 organisms, and the javanica group corresponded to our group 2 organisms. No representatives of the ranarum group were included in our study. Brendle's second major class consisted of the saprophytic strains, which were also split into 3 groups. One of these groups,

TABLE 1. DNA restriction fragment polymorphism determined by intergroup hybridization[a]

Leptospira serovars or strains[b]	Sizes of highly conserved genomic DNA fragments expressed [kb (±SD)][c]												
Group 1													
biflexa	**15.80** (±1.93)		**6.23** (±0.17)	**5.34** (±0.03)									
Group 2													
ballum	**11.50** (±1.65)			**5.19** (±0.20)				2.45 (±0.18)	***2.01*** (±0.09)		1.42 (±0.05)	***1.10*** (±0.07)	0.77 (±0.02)
tarassovi			**6.30** (±0.31)		***4.45*** (±0.20)	**3.68** (±0.13)		***2.48*** (±0.16)				**1.14** (±0.15)	0.81 (±0.02)
hardjob			**6.00** (±0.37)		**4.30** (±0.23)			**2.50** (±0.12)			**1.47** (±0.06)	1.27 (±0.02)	0.71 (±0.03)
Group 3													
hardjop	**11.89** (±0.74)	8.70 (±0.57)		5.33 (±0.24)	4.57 (±0.19)	**3.71** (±0.11)	3.35 (±0.04)	2.47 (±0.02)	**1.98** (±0.04)		***1.40*** (±0.05)		0.73 (±0.04)
australis			6.44 (±0.21)	**5.80** (±0.14)	4.10 (±0.09)	**3.77** (±0.13)	3.41 (±0.05)	2.91 (±0.07)	2.15 (±0.06)	**1.72** (±0.19)	***1.40*** (±0.06)		0.73 (±0.06)
bratislava				**5.58** (±0.38)		**3.75** (±0.14)	3.14 (±0.13)	2.65 (±0.18)		1.85 (±0.05)	***1.43*** (±0.08)	1.06 (±0.09)	**0.74** (±0.07)
pomona				**5.78** (±0.35)	4.58 (±0.41)	**3.75** (±0.21)		2.26 (±0.12)	2.09 (±0.08)	1.75 (±0.09)	1.45 (±0.00)	***1.13*** (±0.07)	**0.76** (±0.06)
canicola				**5.70** (±0.35)	4.45 (±0.27)	**3.80** (±0.09)		2.50 (±0.16)	2.18 (±0.07)			***1.13*** (±0.04)	**0.74** (±0.02)
ictero				**5.70** (±0.29)		**3.81** (±0.07)		2.63 (±0.10)	2.16 (±0.08)		***1.42*** (±0.09)		**0.71** (±0.03)
grippo				5.40 (±0.14)	4.68 (±0.16)	4.13 (±0.08)		2.90 (±0.16)					
autumnalis			**6.07** (±0.43)	**5.02** (±0.32)							**1.42** (±0.10)		0.70 (±0.05)

[a] Boldface numbers, Fragments contain rDNA (not determined for grippotyphosa); italicized numbers, fragments contain 16S rDNA (not determined for biflexa, hardjobovis, grippotyphosa, and autumnalis). Restriction fragments from each leptospira serovar or strain are arranged from left to right in order of decreasing size. Restriction fragments of similar sizes are arranged in columns to emphasize similarities and differences.

[b] Abbreviations: hardjob, hardjobovis; hardjop, hardjoprajitno; ictero, icterohaemorrhagiae; grippo, grippotyphosa.

[c] Mean and standard deviations of 3 to 16 experiments.

represented by Codice, corresponded to our group 1 organism. Brendle's third major class contained only *L. illini*. The DNA in its genome did not hybridize appreciably to genomic DNA from the other leptospiral strains. In our study, there was no detectable hybridization between *L. illini* and any tested leptospiral DNA.

Yasuda et al. (16) also studied DNA relatedness but used binding of DNA duplexes to hydroxyapatite as a criterion to measure homology. They proposed that the leptospiral strains be reclassified into 10 species, 7 of which were new species. Assignments to each of the 10 leptospiral species were based on the extent of DNA cross-hybridization. The assignments made by Yasuda et al. (16) are in agreement with both the data cited above (1, 4) and the results we report in this communication. Our group 1 leptospira (representing serovar biflexa) would belong to the proposed species *Leptospira wolbachii*. Our group 2 leptospira (representing serogroups Ballum and Tarassovi) correspond to the proposed species *Leptospira borgpetersenii*. Our group 3 organisms (representing serogroups Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, and Grippotyphosa) would remain in the existing species, *L. interrogans*, according to the Yasuda proposal. Upon examination of the Sejroe serogroup, Yasuda et al. (16) assigned its members to either *L. borgpetersenii* or *L. interrogans* on the basis of the extent of DNA cross-hybridization. Although we examined different serovars, we also assigned one serovar, hardjo (type hardjobovis), to group 2 and a second serovar, hardjo (type hardjoprajitno), to group 3.

Le Febvre and Thiermann (9) compared genetic relatedness of leptospiral serovars of the Sejroe and Pomona serogroups by DNA cross-hybridization of Southern blots. They demonstrated strong hybridization between pomona and kennewicki, as would be expected with different strains of a serovar. We also observed extensive cross-hybridization between these two strains. Upon examination of Sejroe serovars, Le Febvre and Thiermann (9) found that hardjo strain hardjobovis hybridized extensively with balcanica but exhibited limited cross-hybridization with hardjo strain hardjoprajitno. Instead, hardjoprajitno hybridized more strongly with pomona and kennewicki. These data also agree with our findings.

Our study and the four previous studies were conducted over a period of roughly 20 years, and each laboratory used different, although related, means to assess the relatedness among genomes of various leptospiral species and strains. Together, these studies indicate that the differences in DNA homology among leptospiral strains and species are quite reproducible, irrespective of which method is used to assess relatedness or how long the strains have been maintained in each laboratory. These results justify the conclusion that the leptospira can be identified on the basis of DNA relatedness among their genomes.

Diagnosis of leptospirosis has relied on isolation of leptospira from clinical specimens. This is often not feasible and is a lengthy and costly procedure. As a consequence, the feasibility of dot blot nucleic acid hybridization for diagnosis of leptospirosis has been explored by using genomic DNA from two leptospiral strains differing in G+C content (13). Results of that work suggested that mixed genomic DNA probes could be produced that would give the desired sensitivity for leptospiral DNA detection in clinical speci-

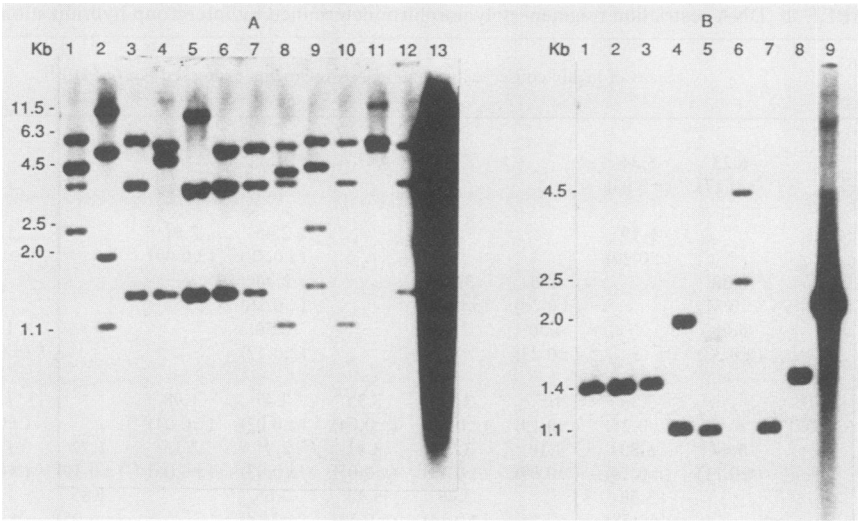

FIG. 3. (A) pNO1300 [^{32}P]DNA was used to probe a Southern blot of *Eco*RI-digested genomic leptospiral DNA. Lanes: 1, tarassovi; 2, ballum; 3, australis; 4, autumnalis; 5, hardjoprajitno; 6, icterohaemorrhagiae; 7, bratislava; 8, canicola; 9, hardjobovis; 10, kennewicki; 11, biflexa; 12, muenchen; 13, pNO1300. (B) pNO1311 [^{32}P]DNA was used to probe a similar Southern blot. Lanes: 1, icterohaemorrhagiae; 2, hardjoprajitno; 3, bratislava; 4, ballum; 5, canicola; 6, tarassovi; 7, pomona; 8, australis; 9, *E. coli*.

mens. Results of our study suggest that selection of the particular leptospiral strains for preparation of a mixed genomic DNA probe to include members of different relatedness groups suspected as infective agents would increase the chance of a given probe successfully identifying leptospiral DNA in a clinical specimen.

In many instances, it would be desirable to know not just whether leptospira were present in a clinical specimen but also the identity of the particular serovar or serogroup. While *L. interrogans* hardjo strain hardjobovis-specific recombinant DNA probes have been developed and are being used to identify clinical infection with this organism (8, 15, 17), the development of specific recombinant probes for a variety of common animal and human pathogens is a formidable task.

The *Eco*RI restriction fragment polymorphism observed with intergroup genomic DNA hybridization could potentially be used to classify leptospira. Each organism listed in Table 1 has either a uniquely sized *Eco*RI restriction fragment or a unique fragment pattern, as compared with the other organisms in the table. Within the Pomona serogroup, however, strains pomona and kennewicki are indistinguishable on the basis of their *Eco*RI restriction fragment pattern. Within the Australis serogroup, serovars lora, jalna, and muenchen cannot be distinguished from bratislava, although serovars bratislava and australis have different *Eco*RI restriction fragment patterns. While more organisms must be studied and compared before the restriction fragment pattern polymorphism can be used diagnostically, the potential for discrimination among serogroups, and possibly serovars, is apparent. If the desired sensitivity could be achieved, genomic DNA from a distantly related leptospira such as *L. biflexa* could be used to probe Southern blots of DNA from clinical specimens. DNA hybridization would indicate not only the presence of leptospiral DNA in a clinical sample but, on the basis of the restriction fragment pattern produced, could give an indication of the potential infecting serogroup or serovar.

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