JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1997, p. 2612–2617 Vol. 35, No. 10
0095-1137/97/$04.00+0

Differentiation of *Leptospira interrogans* Isolates by IS*1500* Hybridization and PCR Assays

RICHARD L. ZUERNER* AND CAROLE A. BOLIN

Leptospirosis Reference Center, Zoonotic Diseases Research Unit, National Animal Disease Center, USDA Agriculture Research Service, Ames, Iowa 50010

Received 14 March 1997/Returned for modification 20 May 1997/Accepted 24 July 1997

Genetic variability among *Leptospira interrogans* (sensu stricto) serovars was assessed by Southern blot hybridization and PCR analyses. The experiments used probes directed to sequences in a recently described insertion element, IS*1500*. Hybridization analysis showed that IS*1500* was present on polymorphic fragments and that differences in these patterns could be used to identify serovars. Hybridization analysis was also useful in discriminating between serovar pomona type kennewicki isolates, making possible the identification of 15 previously unrecognized genetic groups. A PCR assay was developed in which the primers are positioned near the terminal inverted repeats of the element and directed outward. This assay yielded characteristic amplification patterns from isolates, allowing them to be identified. We applied these assays to several new animal isolates of *L. interrogans* from Nicaragua, which recently had an outbreak of human leptospirosis. Three groups of isolates were identified: one strain of serovar pomona type kennewicki and two genetically distinct groups of isolates which may be genetic intermediates between serovars canicola and portlandvere. The IS-based typing assays described should be useful for epidemiological analysis of leptospirosis.

Leptospirosis, caused by pathogenic members of the genus *Leptospira*, is one of the most widespread zoonotic diseases in the world. Leptospirosis occurs in wild and domesticated animals, both of which can be a source of human infection. Rapid determination of the source of infection is critical in limiting the spread of the disease. Potential sources of infection can be determined most easily by determining the serological type (serovar) associated with an outbreak of disease, because certain serovars often associate with specific mammalian hosts. By identifying the serovar associated with an outbreak, it is often possible to predict potential sources of infection and thereby control the spread of the disease. However, some leptospiral serovars (e.g., serovar pomona) appear to adapt well to several mammalian hosts (2) and thus can complicate this analysis.

DNA-based typing is useful for serovar identification because pathogenic leptospires are genetically diverse. Recent reclassification of *Leptospira interrogans* (sensu lato) resulted in recognition of seven distinct species: *L. borgpetersenii*, *L. inadia*, *L. interrogans* (sensu stricto), *L. kirschneri*, *L. noguchii*, *L. santarosai*, and *L. weilii* (20, 27). Because of genetic diversity, serovars can often be identified by characteristic restriction endonuclease digestion patterns (23). While arbitrarily primed PCR provides a sensitive technique for typing pathogenic leptospires (16, 19), it does not offer sensitive detection. Other PCR-based assays typically are useful for sensitive detection of pathogenic leptospires (7, 13, 22, 24) but are of limited usefulness in serovar identification.

To overcome existing limitations in serovar detection and identification, we recently developed a PCR assay that by targeting the insertion sequence IS*1533*, provides a method for sensitive detection and identification of many *L. interrogans* (sensu lato) serovars (30). While many serovars can be identified by this technique, the method is limited because there are few copies of IS*1533* among members of *L. interrogans* (sensu stricto). In the present study, we applied IS-based typing to *L. interrogans* (sensu stricto) by using a newly defined element, IS*1500* (3). In this study, we define several new genetic groups of *L. interrogans* serovar pomona type kennewicki. This is particularly important, because this serovar is commonly isolated from a variety of animal host species (2). The techniques developed were applied to identify and characterize leptospiral strains isolated from a region of Nicaragua that recently had an outbreak of human leptospirosis.

* Corresponding author. Mailing address: Leptospirosis Reference Center, Zoonotic Diseases Research Unit, National Animal Disease Center, USDA Agriculture Research Service, P.O. Box 70, Ames, IA 50010. Phone: (515) 239-8392. Fax: (515) 239-8458. E-mail: rzuerner@nadc.ars.usda.gov.

MATERIALS AND METHODS

Bacterial strains. *L. interrogans* (sensu lato) cells were routinely grown in semisolid or liquid EMJH medium at 30°C (6, 10). Serovar reference strains used in this study are listed in Table 1. Reference isolates of *L. interrogans* serovar pomona type kennewicki are listed in Table 2. Nicaraguan isolates of *L. interrogans* are listed in Table 3.

Preparation of DNA and PCR techniques. Genomic DNA was extracted from *L. interrogans* as described by Thiermann et al. (23). Primers used for this study are shown in Fig. 1. PCRs were carried out with a GeneAmp PCR system 9600 (Perkin-Elmer Norwalk, Conn.) with the reaction conditions recommended by the manufacturer. The temperature cycles used for amplification were 94°C for 30 s, 60°C for 30 s, and 74°C for 2 min, with the extension time increased by 6 s per cycle starting after the sixth cycle for a total of 30 cycles. Sample dye was added to each sample, and the amplification products were separated by electrophoresis through 1.5% NuSieve-GTG agarose gels (3.5:1) (FMC Corp., Rockland, Maine) in 1× TBE (89 mM Tris, 89 mM boric acid, 5 mM EDTA). Products were visualized by UV illumination of the ethidium bromide-stained gel. Genomic DNA from *L. interrogans* serovar pomona strain RZ11 was used as an internal positive control for the assays. Blank samples lacking genomic DNA were used as negative controls.

DNA hybridization analysis. Approximately 3.75 μg of genomic DNA was digested with restriction enzymes and separated by continuous field gel electrophoresis through 0.7% GTG agarose gels buffered with 1× TBE. The DNA was denatured and transferred to a Hybond-N membrane (Amersham Corp., Arlington Heights, Ill.), and hybridization analysis was performed as described previously (32). Two nucleic acid probes were used: pLI17, which contains part of IS*1533* (31); and pI16, which contains part of the internal unique region of IS*1500* (3). Probes were radiolabeled by nick translation as described previously (3) or by random hexanucleotide priming in the presence of [α-^{33}P]dATP (10 μCi/μl [3,000 Ci/mmol]; ICN Radiochemicals, Irvine, Calif.) with a commercially available kit (Amersham Corp.). Filters were used to expose BIOMAX AR film (Kodak Corp., Rochester, N.Y.) at −80°C.

TABLE 1. *Leptospira* serovar reference strains

Serovar	Strain	Species designation
Serogroup Canicola		
bafani	Bafani	*L. kirschneri*
benjamini	Benjamin	*L. interrogans* (sensu stricto)
bindjei	Bindjei	*L. interrogans* (sensu stricto)
broomi	Patane	*L. interrogans* (sensu stricto)
canicola	Hond Utrecht IV	*L. interrogans* (sensu stricto)
galtoni	Lt 1014	*L. kirschneri*
jonsis	Jones	*L. interrogans* (sensu stricto)
kamituga	Kamituga	*L. kirschneri*
kuwait	136/3/3	Unknown
malaya	H6	*L. inadia*
portlandvere	My1039	*L. interrogans* (sensu stricto)
schueffneri	Vleermuis 90c	*L. interrogans* (sensu stricto)
sumneri	Sumner	*L. interrogans* (sensu stricto)
Serogroup Pomona		
kunming	K5	*L. kirschneri*
monjakov	Monjakov	*L. interrogans* (sensu stricto)
mozdok	5621	*L. kirschneri*
pomona	Pomona (type pomona)	*L. interrogans* (sensu stricto)
	LT1026 (type kennewicki)	*L. interrogans* (sensu stricto)
proechimys	1161 U	*L. noguchi*
tropica	CZ 299U	*L. santarosai*
tsaratsova	B81/7	Unknown

RESULTS

DNA hybridization analysis of serovar pomona type kennewicki isolates. We recently reported that strains of *L. interrogans* serovar pomona type kennewicki exhibit a pattern of genetic variability that correlates with the animal host species from which they were isolated (2). To learn if the restriction fragment length polymorphisms (RFLPs) previously described occur near known species of leptospiral IS elements (i.e., IS*1500* or IS*1533*), we characterized our collection of kennewicki isolates by Southern blot hybridization analysis with IS-specific probes.

Hybridization of DNA from various serovar pomona type kennewicki isolates with a probe directed toward the newly defined element IS*1500* (3) showed that these isolates have considerable genetic variability. Restriction enzymes *Hae*III and *Hha*I were the most useful for detecting RFLPs among these isolates. Examples of the different hybridization patterns obtained with *Hae*III or *Hha*I digested DNA are shown in Fig. 2A and B, respectively. By screening DNA from our serovar pomona type kennewicki collection ($n = 81$), we found that each isolate could be clustered into one of 15 groups based on distinct hybridization patterns. These data are summarized in Table 2. Variation in the IS*1533* hybridization patterns was also tested, because this element is commonly associated with RFLPs in many pathogenic leptospiral serovars (32). We found no differences in the hybridization patterns among the isolates when using a probe specific for IS*1533* (data not shown). Neither IS element was associated with the RFLP patterns associated with isolation from different host species (2).

Pulsed-field gel electrophoresis (PFGE) has been useful in differentiating serovars (8, 9) and in detecting rearrangements in *L. borgpetersenii* serovar hardjo (33). We compared DNAs from several serovar pomona type kennewicki isolates representing different genetic groups by restriction endonuclease digestion and PFGE. We did not detect differences in the restriction endonuclease digestion patterns of the isolates tested by PFGE (data not shown).

Differentiation of *L. interrogans* serovars by IS*1500* hybridization analysis. Because the IS*1500* probe was useful in detecting genetic variation among serovar pomona isolates, we predicted that this probe would also be useful in differentiating *L. interrogans* serovars. Results from comparison of representative members of serogroup Pomona by hybridization analysis are shown in Fig. 3. The IS*1500* probe hybridized with serovars pomona and monjakov (*L. interrogans*), serovar proechimys (*L. noguchi*), and serovar tropica (*L. santarosai*). Serovars kunming and mozdok (*L. kirschneri*) did not hybridize with IS*1500*. The IS*1500* hybridization patterns for each serovar were different.

Development of PCR assays. To improve the ease with which IS*1500*-based typing could be done, we sought to develop PCR-based assays that could detect this element and could differentiate serovars. Initially, we screened DNA from our collection of reference strains [$n = 170$ *L. interrogans* (sensu lato) serovars] to assess the range of serovars in which IS*1500* could be detected. For this purpose, we screened the collection of serovar type strain DNA with the primer pair P1 and M16, which direct amplification of an internal fragment of

TABLE 2. *L. interrogans* serovar pomona type kennewicki isolates used for reference

Strain	Host	Geographical region	RFLP pattern[a]		Genetic group
			*Hae*III	*Hha*I	
025	Cattle	South Dakota	1	1	I
009	Cattle	Indiana	2	2	II
020	Cattle	New York	10	2	III
024	Human	Iowa	3	3	IV
027	Cattle	Iowa	4	4	V
034	Cattle	Florida	5	5	VI
042	Swine	Iowa	6	6	VII
049	Swine	Chile	7	7	VIII
050	Cattle	Iowa	7	1	IX
062	Cattle	Florida	9	2	X
063	Cattle	Florida	9	5	XI
078	Cattle	Oklahoma	9	1	XII
102	Cattle	Tennessee	8	9	XIII
080	Cattle	Florida	9	8	XIV
101	Cattle	Texas	11	10	XV

[a] Determined by pI16 probe hybridization.

TABLE 3. Nicaraguan isolates of *L. interrogans*

Strain	Animal host	Genetic identification[a]
862	Swine	Serovar pomona type kennewicki genetic group III
CSD2	Dog	Intermediate to serovars canicola and portlandvere
CSD3	Dog	Intermediate to serovars canicola and portlandvere
CSD5	Dog	Intermediate to serovars canicola and portlandvere
CSD7[b]	Dog	Intermediate to serovars canicola and portlandvere
CSD10[b]	Dog	Intermediate to serovars canicola and portlandvere
1880	Mouse	Intermediate to serovars canicola and portlandvere

[a] Results are based on patterns of restriction fragments visualized by ethidium bromide staining, pI16 probe hybridization, and PCR.
[b] Isolates CSD7 and CSD10 have a hybridization pattern in common that is different from those of other isolates or reference strains.

IS*1500*. DNA from nearly all serovars yielded amplification products with these primers, but we detected the predicted 660-bp fragment from reaction mixtures containing DNA from 123 of 170 serovars tested. Some of these P1-plus-M16-directed amplicons of different sizes are probably partial IS*1500*-like elements (see Discussion).

Using the inverse complement of primers P1 and M16 (iP1 and iM16, respectively), a PCR-based typing assay was developed. These primers direct amplification outward from IS*1500* to sequences that flank the elements that are between two closely placed copies of IS*1500*. This strategy is similar to one used successfully with IS*1533* (30). The iP1 and iM16 primers were tested with DNA from representatives of serogroup Pomona (Fig. 4). Each serovar yielded a novel, complex amplification pattern with either the complete primer pair or each primer alone. Subsequent analysis of DNA from serovar pomona isolates showed that the PCR-based assay was unable to differentiate between isolates belonging to the same serovar (data not shown). Generation of these reaction products was consistent and reproducible.

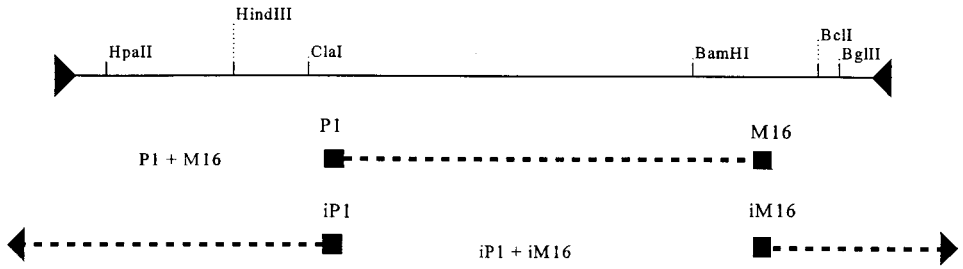
Analysis of leptospiral isolates from a Nicaraguan outbreak. We sought to identify seven animal strains of *Leptospira* obtained from an area of rural Nicaragua shortly after an outbreak of human leptospirosis in November 1995 (28).

One leptospiral isolate from Nicaragua (isolate 862) was identified as *L. interrogans* serovar pomona type kennewicki by the combined techniques of restriction endonuclease analysis, PCR (data not shown), and IS*1500* hybridization (Fig. 2). With the hybridization data, it was determined that isolate 862 belonged to genetic group III (Table 2).

Six strains had restriction endonuclease digestion patterns consistent with *L. interrogans* serovar portlandvere, being differentiated from serovar canicola by the presence of a single restriction fragment approximately 15 kb in size (Fig. 5). The iP1 plus iM16 amplification pattern of these strains was compared with those of representatives of serogroup Canicola (Fig. 6). The amplification patterns obtained with DNA from serovars canicola and portlandvere were quite similar, being differentiated by minor products with sizes of about 517 bp (present in serovar portlandvere) and 950 bp (present in serovar canicola) (Fig. 6, lanes 12 and 13). The amplification products from this set of isolates is similar to both serovars canicola and portlandvere, having both the 517- and 950-bp amplification products (Fig. 6, lanes 14 to 19). These isolates were characterized further by hybridization analysis with the IS*1500* probe. We found that the hybridization patterns of DNA from serovars canicola and portlandvere are similar, with the exception of an extra fragment in serovar canicola with a size about 3.2 kbp which often comigrates with the 3.1-kbp fragment common to both serovars (Fig. 7). The hybridization patterns of all but two isolates (CSD7 and CSD10) were identical to those of serovar portlandvere (Fig. 7). Strains CSD7 and CSD10 both appear to have an extra copy of IS*1500* that is seen in both *Hae*III and *Hha*I digests (Fig. 7). These data are summarized in Table 3.

DISCUSSION

This study describes the development of improved methods for the identification and differentiation of *L. interrogans* (sensu stricto) isolates. Because *L. interrogans* infections are per-



Name	Primer Sequence (5' to 3')	Position
P1	TTC-GAT-TCA-AAG-CAT-GGC-TAA-CG	386
M16	AAA-GAA-GGA-CTC-AGC-GAC-TGC-G	1046 (C)
iP1	CGT-TAG-CCA-TGC-TTT-GAA-TCG-AA	386 (C)
iM16	CGC-AGT-CGC-TGA-GTC-CTT-CTT-T	1046

FIG. 1. Primers used for PCR. A schematic of IS*1500* is shown. The triangles at the ends of the element represent the terminal inverted repeats The locations of the primers (squares) and amplified products (dashed lines) are shown. Arrows indicate the direction of DNA synthesis during the PCRs from the outward-facing primers. Primer sequences and their positions in the element are presented below. The C designation (in parentheses) indicates that the primer is positioned on the complementary strand of DNA (i.e., in the 3′-to-5′ direction) with respect to the diagram.

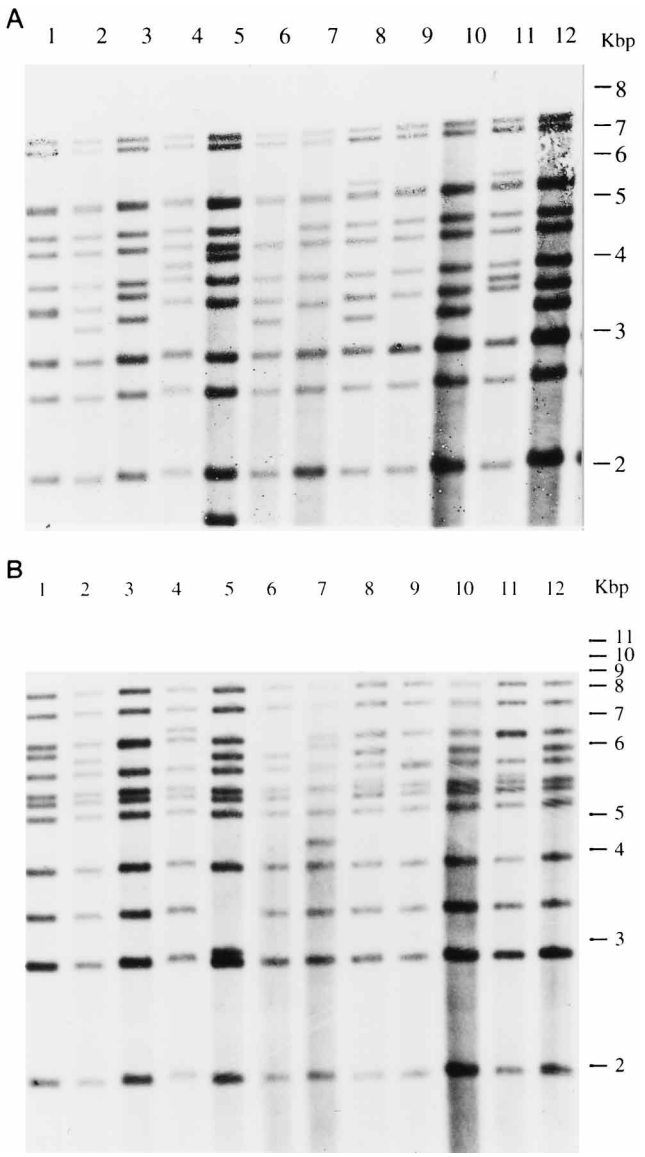


FIG. 2. IS*1500* hybridization patterns with serovar pomona isolates. Genomic DNAs of serovar pomona isolates were digested with either *Hae*III (A) or *Hha*I (B), separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with pI16. The samples represented are strains 025 (lane 1), 009 (lane 2), 024 (lane 3), 027 (lane 4), 034 (lane 5), 042 (lane 6), 049 (lane 7), 102 (lane 8), 080 (lane 9), 020 (lane 10), 101 (lane 11), and 862 (lane 12). The autoradiographs are shown. The migration of size standards is shown on the left.

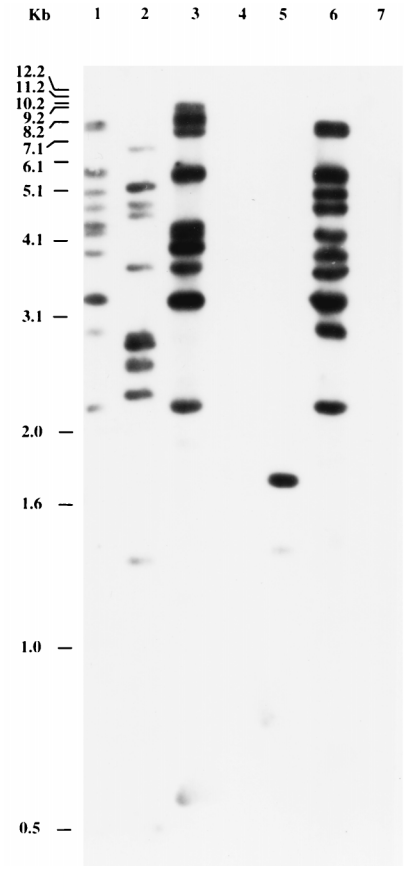


FIG. 3. IS*1500* hybridization analysis of representative serogroup Pomona type strains and Nicaraguan isolate 862. Genomic DNA was digested with *Hae*III and processed as described for Fig. 2. The samples represented are strains pomona (lane 1), 1161U (lane 2), monjakov (lane 3), 5621 (lane 4), CZ299U (lane 5), LT1026 (lane 6), and K5 (lane 7). The autoradiograph is shown. The migration of size standards is shown on the left.

vasive in wildlife populations and leptospirosis is considered a reemerging human disease, rapid identification of serovars is essential to localize and contain potential animal sources of human infection. Specific serovars are often associated with specific animal species (e.g., serovars canicola and portlandvere are most commonly isolated from dogs). Thus, serovar identification can often provide important clues about the potential animal sources of infection. However, serological characterization of isolates often shows considerable variation between laboratories, is cumbersome, and is quite slow. We have sought to develop rapid serovar identification methods based on genetic typing, exploiting the genetic variability that often exists between serovars for differentiation. Using the techniques developed in this study, we identified new genetic variants in serovar pomona and have identified possible genetic intermediates to serovars canicola and portlandvere. The presence of genetic variation among isolates is useful in epidemiological analysis, because we can identify differences within otherwise homogeneous groups of isolates.

A concern that led to the development of these assays was to

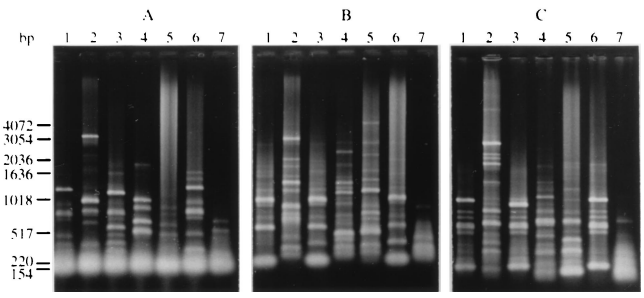


FIG. 4. PCR analysis of serogroup Pomona. Genomic DNA was amplified by PCR with the primers iP1 plus iM16 (A), iP1 alone (B), or iM16 alone (C) and separated by agarose gel electrophoresis. The samples represented are strains pomona (lane 1), 1161U (lane 2), monjakov (lane 3), 5621 (lane 4), CZ299U (lane 5), LT1026 (lane 6), and B81/7 (lane 7). The ethidium bromide gel is shown. The migration of size standards is shown on the left.

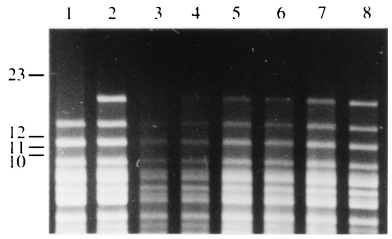


FIG. 5. Restriction endonuclease analysis of serovars canicola and portlandvere and comparison with Nicaraguan isolates. Samples of genomic DNA were digested with *Hha*I and separated by agarose gel electrophoresis. The samples represented are serovar canicola strain Hond Utrecht IV (lane 1), serovar portlandvere strain MY1039 (lane 2), isolate CSD2 (lane 3), isolate CSD3 (lane 4), isolate CSD5 (lane 5), isolate CSD7 (lane 6), isolate CSD10 (lane 7), and isolate 1880 (lane 8). The ethidium bromide-stained gel is shown. The migration of size standards is shown on the left in kilobase pairs.

overcome the limitations of a previously developed PCR-based assay that targets IS*1533* sequences (30). That assay requires low annealing temperatures when used with isolates from several diverse pathogenic *Leptospira* species. In addition, several serovars have few full or partial copies of IS*1533*, thus making detection or identification difficult. The IS*1500*-based assays developed here complement the IS*1533*-based techniques and extend the number of serovars that can be analyzed. Genomic DNA from some serovars tested with the IS*1500* assays failed to yield either amplification products or detectable hybridization patterns. However, the IS*1500*-based assay is very useful in detecting and identifying *L. interrogans* (sensu stricto) isolates, which the IS*1533*-based assay does poorly. We note that IS*1500* is distinct from an element described by Savio et al. (22 [also see reference 3 and unpublished data]).

Bacterial typing with assays based on IS elements has been shown to have considerable epidemiological value (11, 12, 15, 17, 21, 25, 32, 33). We have shown previously that characterization of leptospiral isolates with IS-based assays provides information about the extent of genetic diversity that exists within different serovars (32, 33). This information has been useful for identifying genetic variants that also exhibit different patterns of infection (1) and that thereby help to identify

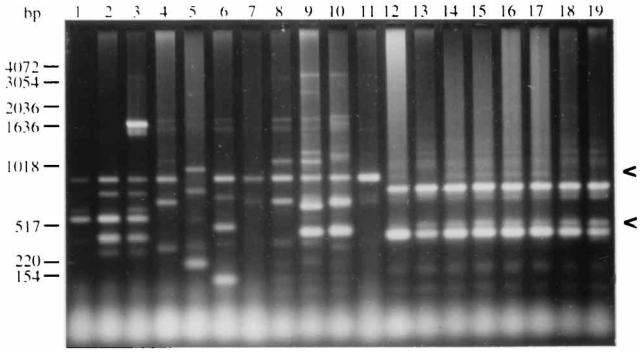


FIG. 6. PCR analysis of serogroup Canicola and Nicaraguan isolates. Genomic DNA was amplified by PCR with the primers iP1 plus iM16 and separated by agarose gel electrophoresis. The samples represented are strains LT1014 (lane 1), Bafani (lane 2), Kamituga (lane 3), Jones (lane 4), Sumner (lane 5), Patane (lane 6), Bindjei (lane 7), Vleermuis 90C (lane 8), Benjamin (lane 9), H6 (lane 10), 136/3/3 (lane 11), Hond Utrecht IV (lane 12), MY1039 (lane 13), CSD2 (lane 14), CSD3 (lane 15), CSD5 (lane 16), CSD7 (lane 17), CSD10 (lane 18), and 1880 (lane 19). The ethidium bromide-stained gel is shown. The migration of size standards is shown on the left. Arrows on the right indicate the two minor bands which are common to the Nicaraguan isolates, as discussed in the text.

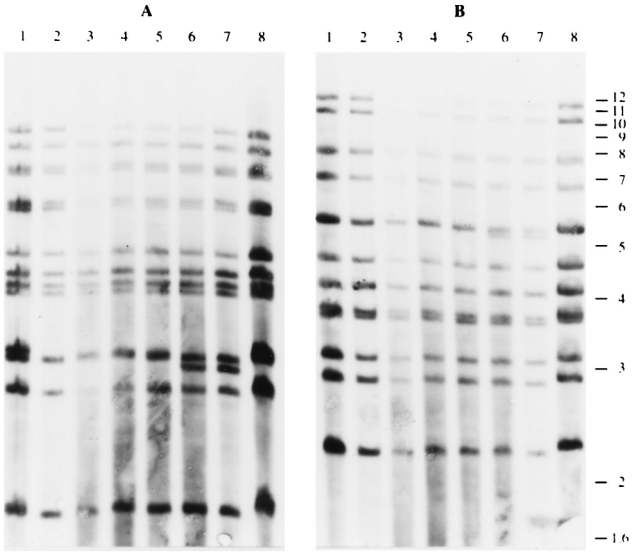


FIG. 7. IS*1500* hybridization analysis of Nicaraguan isolates. Samples of genomic DNA were digested with *Hae*III (A) or *Hha*I (B) and processed as described for Fig. 2. The samples represented are serovar canicola strain Hond Utrecht IV (lane 1), serovar portlandvere strain MY1039 (lane 2), isolate CSD2 (lane 3), isolate CSD3 (lane 4), isolate CSD5 (lane 5), isolate CSD7 (lane 6), isolate CSD10 (lane 7), and isolate 1880 (lane 8). The autoradiograph obtained after hybridization with pI16 is shown. The migration of size standards is shown on the left in kilobase pairs.

differences in virulence traits among quite similar strains. The success of the iP1 and iM16 primers in amplifying products and the appearance of several aberrant products with P1 and M16 primers suggest that IS*1500* likely undergoes a variety of deletions. Although the nucleotide sequences of these products were not determined, we predict that they are probably partial IS elements, similar to what was found with IS*1533* (30). Previously, we showed that the *L. interrogans* genome is quite fluid, with large chromosomal rearrangements occurring between closely related serovars (32, 34). Regions of the genome near the ends of these rearrangements often have copies of IS elements, and these elements may play a role in the flexibility of the genome (3). Such rearrangements may generate partial elements that in turn allow the outward-facing primers to be useful in typing the bacteria.

Leptospirosis caused by serovar pomona is a persistent problem in the cattle industry (14, 18) and was recently associated with equine abortion (4, 26) and uveitis and blindness (5). Although we predicted that either IS*1533* or IS*1500* might be closely linked with a set of RFLPs associated with the host species from which they are isolated (2), such a relationship could not be shown. However, we detected 15 new genetic groups of serovar pomona type kennewicki that were previously unknown. The host species-associated RFLPs may occur by mechanisms other than recombination between copies of IS*1500* or IS*1533*. The *L. interrogans* genome has several other, yet uncharacterized repetitive DNA elements (29) and possibly another distinct and yet unnamed IS-like element (22). Three hybridization groups of kennewicki isolates are of particular interest. Group I isolates are seen most frequently, are isolated from all geographic locations sampled, and may represent the more successful pathogens of this serovar. Strain 027 (genetic group V) has the same restriction endonuclease pattern as type kennewicki isolates, but it has a hybridization pattern identical to serovar pomona type pomona, and may represent an intermediate between the known types of serovar pomona. Isolates

having *Hha*I pattern 7 were seen only in isolates from Chile, suggesting the emergence of a clonal population.

Many isolates from Nicaragua were genetically similar to both serovars canicola and portlandvere. The restriction endonuclease patterns of the isolates were identical to those of serovar portlandvere, whereas the PCR results suggest these isolates may be genetic intermediates between serovars canicola and portlandvere. This analysis identified genetic differences between the isolates, and these differences may be useful in epidemiological analysis of human infections from this region.

The ability to discriminate between isolates by using IS*1500* will provide a useful tool in epidemiology. It will also allow us to focus future pathogenesis studies on genetically different strains of serovar pomona. As we reported previously, IS-based typing of *L. borgpetersenii* serovar hardjo was useful in identifying different genetic groups (33), which were subsequently found to present different clinical signs during experimental infections (1). We expect that IS-based typing of serovar pomona type kennewicki will also be useful in helping to define differences among the pathogenic potentials of different strains.

ACKNOWLEDGMENTS

We thank Wendy Johnson, Annette Olsen, and John Foley for technical assistance and David Ashford and Robbin Weyant for assistance in collecting the isolates.

REFERENCES

1. **Bolin, C. A., D. P. Alt, J. A. Wagennar, and R. L. Zuerner.** 1994. Experimental infection of cattle with *Leptospira borgpeterenii* serovar hardjo type hardjo-bovis from different countries, p. 41. *In* Proceedings of the VIIIth Meeting of European Leptospiral Workers. Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Rome, Italy.
2. **Bolin, C. A., and R. L. Zuerner.** 1996. Correlation between DNA restriction fragment length polymorphisms in *Leptospira interrogans* serovar pomona type kennewicki and host animal source. J. Clin. Microbiol. **34:**424–425.
3. **Boursaux-Eude, C., I. Saint Girons, and R. L. Zuerner.** 1995. Characterization of IS*1500*, an IS*3*-like element isolated from *Leptospira interrogans*. Microbiology **141:**2165–2173.
4. **Donahue, J. M., B. J. Smith, K. B. Poonacha, J. K. Donahoe, and C. L. Rigsby.** 1995. Prevalence and serovars of leptospira involved in equine abortions in central Kentucky during the 1991–1993 foaling seasons. J. Vet. Diagn. Invest. **7:**87–91.
5. **Dwyer, A. E., R. S. Crockett, and C. M. Kalsow.** 1995. Association of leptospiral seroreactivity and breed with uveitis and blindness in horses: 372 cases (1986–1993). Am. J. Vet. Med. Assoc. **207:**1327–1331.
6. **Ellinghausen, H. C., and W. G. McCullough.** 1965. Nutrition of *Leptospira* pomona and growth of 13 other serotypes: fractionation of oleic albumin complex and medium bovine albumin and polysorbate 80. Am. J. Vet. Res. **26:**45–51.
7. **Gravekampp, C., H. van de Kemp, M. Franzen, D. Carrington, G. J. Schoone, G. J. J. M. van Eys, C. O. R. Everard, R. A. Hartskeerl, and W. J. Terpstra.** 1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J. Gen. Microbiol. **139:**1691–1700.
8. **Herrmann, J. L., C. Baril, E. Bellenger, P. Perolat, G. Baranton, and I. Saint Girons.** 1991. Genome conservation in isolates of *Leptospira interrogans*. J. Bacteriol. **173:**7582–7588.
9. **Herrmann, J. L., E. Bellenger, P. Perolat, G. Baranton, and I. Saint Girons.** 1992. Pulsed-field gel electrophoresis of *Not*I digests of leptospiral DNA: a new rapid method of serovar identification. J. Clin. Microbiol. **30:**1696–1702.
10. **Johnson, R. C., and V. G. Harris.** 1967. Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. J. Bacteriol. **94:**27–31.
11. **Lawrence, J. G., D. E. Dykhuizen, R. F. DuBose, and D. L. Hartl.** 1989. Phylogenetic analysis using insertion sequence fingerprinting in *Escherichia coli*. Mol. Biol. Evol. **6:**1–14.
12. **Mazurek, G. H., M. D. Cave, K. D. Eisenach, R. J. Wallace, Jr., J. H. Bates, and J. T. Crawford.** 1991. Chromosomal DNA fingerprint patterns produced with IS*6110* as strain-specific markers for epidemiological study of tuberculosis. J. Clin. Microbiol. **29:**2030–2033.
13. **Mérien, F., P. Amouriaux, P. Perolat, G. Baranton, and I. Saint Girons.** 1992. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. J. Clin. Microbiol. **30:**2219–2224.
14. **Miller, D. A., M. A. Wilson, and G. W. Beran.** 1991. Survey to estimate prevalence of *Leptospira interrogans* infection in mature cattle in the United States. Am. J. Vet. Res. **52:**1761–1765.
15. **Pacciarini, M. L., M. L. Savio, S. Tagliabue, and C. Rossi.** 1992. Repetitive sequences cloned from *Leptospira interrogans* serovar hardjo genotype hardjoprajitno and their application to serovar identification. J. Clin. Microbiol. **30:**1243–1249.
16. **Perolat, P., F. Merien, W. A. Ellis, and G. Baranton.** 1994. Characterization of *Leptospira* isolates from serovar hardjo by ribotyping, arbitrarily primed PCR, and mapped restriction site polymorphisms. J. Clin. Microbiol. **32:** 1949–1957.
17. **Plikaytis, B. B., J. T. Crawford, C. L. Woodley, W. R. Butler, K. D. Eisenach, M. C. Cave, and T. M. Shinnick.** 1993. Rapid, amplification-based fingerprinting of *Mycobacterium tuberculosis*. J. Gen. Microbiol. **139:**1537–1542.
18. **Prescott, J. F., R. B. Miller, V. M. Nicholson, S. W. Martin, and T. Lesnick.** 1988. Seroprevalence and association with abortion of leptospirosis in cattle in Ontario. Can. J. Vet. Res. **52:**210–215.
19. **Ralph, D., M. McClelland, J. Welsh, G. Baranton, and P. Perolat.** 1993. *Leptospira* species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. J. Bacteriol. **175:**973–981.
20. **Ramadass, P., B. D. W. Jarvis, R. J. Corner, D. Penny, and R. B. Marshall.** 1992. Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. Int. J. Syst. Bacteriol. **42:**215–219.
21. **Rappuoli, R., M. Perugini, and G. Ratti.** 1987. DNA element of *Corynebacterium diphtheriae* with properties of an insertion element and usefulness for epidemiological studies. J. Bacteriol. **169:**308–312.
22. **Savio, M. L., C. Rossi, P. Fusi, S. Tagliabue, and M. L. Pacciarini.** 1994. Detection and identification of *Leptospira interrogans* serovars by PCR coupled with restriction endonuclease analysis of amplified DNA. J. Clin. Microbiol. **32:**935–941.
23. **Thiermann, A. B., A. L. Handsaker, S. L. Moseley, and B. Kingscote.** 1985. New method for classification of leptospiral isolates belonging to serogroup pomona by restriction endonuclease analysis: serovar *kennewicki*. J. Clin. Microbiol. **21:**585–587.
24. **Van Eys, G. J. J. M., C. Gravekamp, M. J. Gerritsen, W. Quint, M. T. E. Cornelissen, J. ter Schegget, and W. J. Terpstra.** 1989. Detection of leptospires in urine by polymerase chain reaction. J. Clin. Microbiol. **27:**2258–2262.
25. **van Soolingen, D., P. W. M. Hermans, P. E. W. de Haas, D. R. Soll, and J. D. A. van Embden.** 1991. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J. Clin. Microbiol. **29:**2578–2586.
26. **Williams, D. M., B. J. Smith, J. M. Donahue, and K. B. Poonacha.** 1994. Serological and microbiological findings on 3 farms with equine leptospiral abortions. Equine Vet. J. **26:**105–108.
27. **Yasuda, B. H., A. G. Steigerwalt, K. R. Sulzer, A. F. Kaufmann, F. Rogers, and D. J. Brenner.** 1987. Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. Int. J. Syst. Bacteriol. **37:**407–415.
28. **Zaki, S. R., W.-J. Shieh, and the Epidemic Working Group.** 1996. Leptospirosis associated with outbreak of acute febrile illness and pulmonary haemorrhage, Nicaragua, 1995. Lancet **347:**535–536.
29. **Zuerner, R. L.** 1993. Genomic structure, organization, and variation in *Leptospira*. J. Cell. Biochem. **17E:**309.
30. **Zuerner, R. L., D. Alt, and C. A. Bolin.** 1995. IS*1533*-based PCR assay for identification of *Leptospira interrogans* sensu lato serovars. J. Clin. Microbiol. **33:**3284–3289.
31. **Zuerner, R. L., and C. A. Bolin.** 1988. Repetitive sequence element cloned from *Leptospira interrogans* serovar hardjo type hardjo-bovis provides a sensitive diagnostic probe for bovine leptospirosis. J. Clin. Microbiol. **26:**2495–2500.
32. **Zuerner, R. L., and C. A. Bolin.** 1990. Nucleic acid probe characterizes *Leptospira interrogans* serovars by restriction fragment length polymorphisms. Vet. Microbiol. **24:**355–366.
33. **Zuerner, R. L., W. A. Ellis, C. A. Bolin, and J. M. Montgomery.** 1993. Restriction fragment length polymorphisms distinguish *Leptospira borgpetersenii* serovar hardjo type hardjo-bovis isolates from different geographical locations. J. Clin. Microbiol. **31:**578–583.
34. **Zuerner, R. L., J.-L. Herrmann, and I. Saint Girons.** 1993. Comparison of genetic maps for two *Leptospira interrogans* serovars provides evidence for two chromosomes and intraspecies heterogeneity. J. Bacteriol. **175:**5445–5451.