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Restriction Fragment Length Polymorphisms Distinguish *Leptospira borgpetersenii* Serovar hardjo Type hardjo-bovis Isolates from Different Geographical Locations

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Genetic variability among *Leptospira borgpetersenii* serovar hardjo type hardjo-bovis isolates representing several geographical regions was determined by restriction endonuclease analysis. Five previously unidentified *Eco*RI digestion patterns and one previously unidentified *Hha*I digestion pattern were seen with the various isolates. The copy number and genomic distribution of an *L. borgpetersenii* insertion sequence (IS*1533*) was determined. Hardjo-bovis isolate 033 (the type strain for hardjo-bovis) contained 40 well dispersed copies of IS*1533*. IS*1533* probes were used to compare hardjo-bovis isolates by DNA blot hybridization analysis. Use of these probes showed the presence of additional genetic heterogeneity among hardjo-bovis isolates, which restriction endonuclease analysis did not show. Pulsed-field gel electrophoretic analysis of DNAs from several isolates suggested that some polymorphisms arose by genomic rearrangements. All hardjo-bovis isolates were categorized into 14 distinct groups on the basis of common hybridization and endonuclease digestion patterns. Most of these groups were isolated from distinct geographical regions, suggesting that several different clonal populations of hardjo-bovis exist.

Leptospira species belonging to serovar hardjo are common etiological agents of bovine leptospirosis (1, 5, 8, 26). These bacteria also cause a common zoonosis in humans (8, 13, 25). Serovar hardjo comprises two species of *Leptospira*: *Leptospira borgpetersenii* serovar hardjo type hardjo-bovis and *Leptospira interrogans* serovar hardjo type hardjoprajitno (the reference strain for serovar hardjo [19]). Hardjo-bovis and hardjoprajitno are usually differentiated by restriction endonuclease analysis (REA) (28) but can also be differentiated by DNA hybridization (30, 34). Hardjo-bovis and hardjoprajitno infections are clinically identical, with the most common signs being reproductive failure in cattle and influenza-like symptoms in humans (4, 5, 8). The geographical distributions of hardjo-bovis and hardjoprajitno differ. The known geographical range of hardjoprajitno is poorly understood, with confirmed isolations having been made only in the British Isles (6) and Indonesia (8). Hardjo-bovis has a broad distribution and, thus, is more commonly associated with bovine leptospirosis. Confirmed isolations of hardjo-bovis have been made in North America (28), the British Isles (6), mainland Europe (30), New Zealand (6), and Australia (24).

Although hardjo-bovis is frequently detected in cattle herds throughout the world, the occurrence of hardjo-bovis-caused cattle abortions and zoonotic infection of humans varies among different geographical regions. In North America, hardjo-bovis often causes reproductive failure in cattle while human infections are uncommon (15, 18, 27). In the British Isles, clinical disease occurs in cattle and humans and can be caused by either hardjo-bovis or hardjoprajitno (4, 5, 8). Clinical hardjo-bovis infections occur throughout mainland Europe, but human infections are detected primarily in the Netherlands (8). Hardjo-bovis is not commonly associated with bovine abortions in Australia and New Zealand (2), while it is a common cause of human leptospirosis there (13, 25). In South America, serovar hardjo is prevalent and is often associated with herds which have experienced abortion storms (1). However, in South America, leptospiral serovars other than hardjo are more common in clinical human disease (3). Although serovar hardjo has been associated with cattle abortions in Africa (26), the frequency of serovar hardjo infections in either cattle or humans in Africa is not known. The genetic types of serovar hardjo prevalent in Africa or South America have not been reported.

Although several features may be involved, differences in the epidemiology of hardjo-bovis infections suggest that hardjo-bovis isolates from diverse geographical regions may differ at the genetic level. Evidence for genetic heterogeneity among hardjo-bovis isolates has been shown with REA (28). Three distinct types (A, B, and C) of hardjo-bovis isolates are identified by restriction fragment length polymorphisms (RFLPs) by using restriction enzyme *Hha*I (28). Additional studies using REA to characterize hardjo-bovis isolates have failed to detect any additional genetic heterogeneity (6, 24).

In this report, hardjo-bovis isolates obtained from several different geographical regions were compared by REA and by DNA blot hybridization. On the basis of these results, the known range of hardjo-bovis was extended to include Africa and South America. Hybridization probes from an insertion sequence (IS) element cloned from hardjo-bovis, IS*1533* (33a, 34), were also used to detect genetic differences among hardjo-bovis isolates. IS elements are mobile genetic elements, capable of moving to different places within the genome, and can often promote genetic recombination (7, 12, 21–23). Earlier, IS*1533* probes were found to be useful

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TABLE 1. *L. borgpetersenii* serovar hardjo type hardjo-bovis isolates used in this study[a]

Isolate	Genetic group	Origin	Source[b]
033	1	Nebraska	NADC
045	2	Iowa	NADC
069	3	Chile	S. Riedemann
057	4	Italy	VRL
196	5	Switzerland	L. Corboz
145	6	Northern Ireland	VRL
323	7	Northern Ireland	VRL
046	8	Iowa	NADC
328	9	Switzerland	L. Corboz
330	10	Portugal	VRL
332	11	New Zealand	VRL
067	12	Florida	F. White
075	13	Florida	F. White
197	13	Nebraska	NADC
068	14	Israel	NVSL

[a] Strains used only for RFLP typing are not presented here. All isolates listed were obtained from cattle.

[b] NADC, National Animal Disease Center, Ames, Iowa; VRL, Veterinary Research Laboratories, Belfast, Northern Ireland; NVSL, National Veterinary Services Laboratory, Ames, Iowa.

for typing leptospires by detecting serovar-specific RFLPs (35). Those findings led us to the present study, in which we investigated the possibility that IS*1533* probes may be useful for detecting genetic differences among members of the same serovar.

MATERIALS AND METHODS

Bacterial strains. Representative *L. borgpetersenii* serovar hardjo type hardjo-bovis isolates used in this study are listed in Table 1. All isolates analyzed except two isolates from humans and five isolates from pigs were obtained from cattle. *L. borgpetersenii* isolates were grown at 30°C in bovine serum albumin-Tween 80 medium (11).

Probes. Plasmids pLI16 and pLI17 (34) contain portions of IS*1533*. Plasmid pLI18 contains the 1.1-kb *Hin*PI fragment of pLI16 inserted into pBSM13 (Stratagene Cloning Systems, La Jolla, Calif.). Plasmid pLI20 was derived from pLI18 by deletion of the 0.9-kb *Eco*RI fragment. In plasmids pLI17, pLI18, and pLI20, the inserted DNA is flanked by phage T3 and T7 promoters. Restriction maps of plasmids used in this study are shown in Fig. 1.

Radiolabeled single-stranded RNA probes were synthesized from *Eco*RV-digested pLI17 by runoff transcription by using phage T3 RNA polymerase (Life Technologies Inc., Gaithersburg, Md.) (probe A) or from *Eco*RI-digested pLI20 by using phage T3 RNA polymerase (probe B) (Fig. 1) in the presence of [α-^{32}P]UTP (ICN Radiochemicals, Inc.) as described previously (34).

Gel electrophoresis and DNA blotting. Genomic DNA was extracted as described elsewhere (29, 33). Protocols for restriction endonuclease digestions, agarose gel electrophoresis, and DNA blot hybridization analysis were described previously (33, 34, 35).

RESULTS

Copy number and distribution of IS*1533* in the hardjo-bovis genome. IS*1533* was initially detected by virtue of its high copy number in the hardjo-bovis genome (34). DNA blot hybridization was used to establish the copy number and distribution of IS*1533* in isolate 033 (the type strain for hardjo-bovis [28]). Probe A (from pLI17) and probe B (from pLI20) were made to discern the left- and right-hand sides of IS*1533*, respectively (Fig. 1). These probes were hybridized with isolate 033 DNA cut with enzymes recognizing unique sites in IS*1533* (*Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xmn*I). With this strategy, fragments hybridizing each probe constitute junction fragments with part of IS*1533* and flanking genomic DNA of unknown sequence. Thus, individual copies of IS*1533* were resolved (Fig. 2A and B). By counting the number of fragments which hybridized with probes A and B, it was determined that isolate 033 contained 40 copies of IS*1533*. This is in good agreement with a previous estimate (34). Since the DNA hybridization pattern of isolate 033 kept in continuous culture for over 2 years did not change (data not shown), it appears that the pattern is stable during in vitro cultivation.

Many large restriction fragments hybridized with IS*1533*. This result suggested that IS*1533* is dispersed throughout the genome. To confirm that IS*1533* was well distributed, probe B was hybridized with large (>50-kb) restriction fragments resolved by pulsed-field gel electrophoresis (Fig. 2C). This approach improved resolution of large fragments and confirmed that IS*1533* was well dispersed. Because hardjo-bovis has many IS*1533* copies distributed around the genome, we predicted that IS-based probes would be useful for detecting genetic differences between isolates.

Restriction endonuclease comparison of *L. borgpetersenii* serovar hardjo type hardjo-bovis isolates. Before comparing all 195 hardjo-bovis isolates by DNA blot hybridization using IS*1533* probes, we compared these isolates by REA using enzymes *Eco*RI and *Hha*I. These two enzymes were used to compare hardjo-bovis isolates in previous studies (6, 24, 28). Six different *Eco*RI digestion patterns (types Ea through Ef) were seen with the isolates (Fig. 3). Isolates having the Ea pattern had 6.4-kb and 7.2-kb fragments, which were absent in pattern Eb. The Eb pattern had a unique 5.7-kb *Eco*RI fragment. The Ec pattern had an intensely staining fragment at 5.2 kb, which was absent in other patterns. Only one isolate showed the Ed pattern, which lacked the 8.1-kb

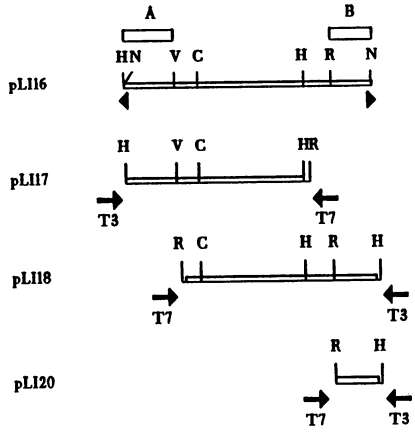


FIG. 1. Restriction maps of plasmids. Restriction maps of plasmids pLI16, pLI17, pLI18, and pLI20 are shown. *L. borgpetersenii* DNA is indicated by a double line. Above the pLI16 map, the positions of probes A and B are shown. The arrowheads below pLI16 show the location of the IS*1533* terminal inverted repeat sequences. The locations and orientations of phage T3 and T7 promoters are shown below plasmids pLI17, pLI18, and pLI20. H, *Hin*dIII; N, NarI; V, *Eco*RV; C, *Cla*I; R, *Eco*RI.

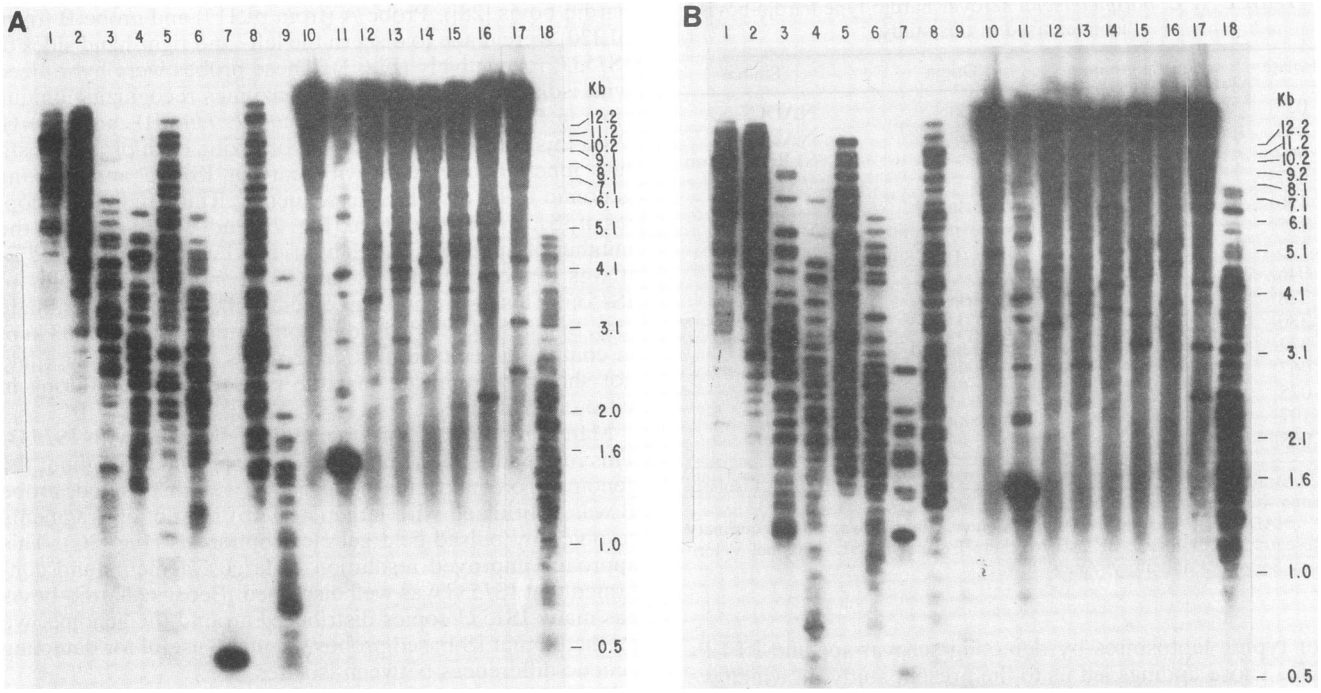


FIG. 2. Copy number and distribution of IS*1533* in hardjo-bovis isolate 033. (A and B) Genomic DNA from hardjo-bovis isolate 033 was digested with *Bam*HI (lanes 1), *Bgl*II (lanes 2), *Cla*I (lanes 3), *Eco*RI (lanes 4), *Eco*RV (lanes 5), *Hha*I (lanes 6), *Hin*dIII (lanes 7), *Nar*I (lanes 8), *Pst*I (lanes 9), *Sac*II (lanes 10), *Sal*I (lanes 11), *Xho*I (lanes 12), or *Xmn*I (lanes 13), separated by constant-field agarose gel electrophoresis, and blotted onto a nylon membrane. (C) Genomic DNA from hardjo-bovis isolate 033 was digested with *Asc*I (lane 1), *Not*I (lane 2), *Pac*I (lane 3), *Srf*I (lane 4), or *Sse*8387I (lane 5), separated by pulsed-field gel electrophoresis, and blotted onto a nylon membrane. Autoradiographs of the membranes after hybridization with probe A (A) or probe B (B and C) are shown.

*Eco*RI fragment. The Ee pattern had a unique 8-kb *Eco*RI fragment. The Ef pattern lacked the 5.3-kb *Eco*RI fragment.

Three different *Hha*I digestion patterns, two (Ha and Hb) that were described previously (28) and one new pattern (Hd), were seen (Fig. 3). The Hc pattern reported for isolate 003 (28) could not be duplicated (data not shown). Type Ha isolates had three fragments (4.9, 5.0, and 7.5 kb), which were absent in the Hb pattern. Type Hb isolates had a unique 6.1-kb *Hha*I fragment. The Hd pattern was similar to Ha, but the 4.9-kb *Hha*I fragment stained with more intensity, suggesting two fragments of similar size comigrating on the gel.

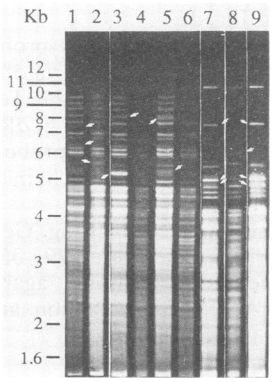
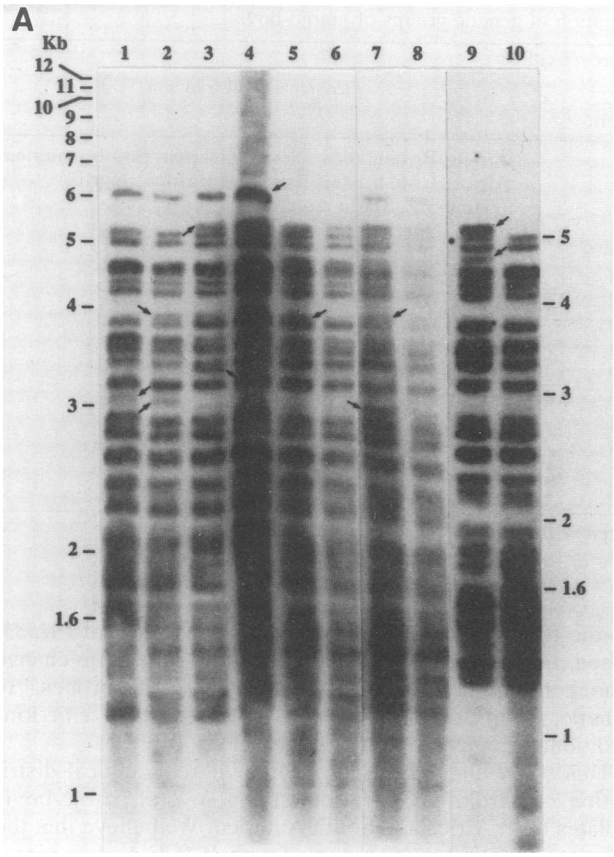


FIG. 3. Restriction endonuclease digestion patterns of hardjo-bovis isolates. Samples of DNAs from hardjo-bovis isolates were digested with *Eco*RI (lanes 1 to 6) or *Hha*I (lanes 7 to 9). *Eco*RI digestion patterns are shown as follows: lane 1, Ea; lane 2, Eb; lane 3, Ec; lane 4, Ed; lane 5, Ee; lane 6, Ef. Lanes 7 to 9, *Hha*I digestion patterns Ha, Hb, and Hd, respectively. The ethidium bromide-stained gel is shown. Arrows indicate distinguishing fragments.



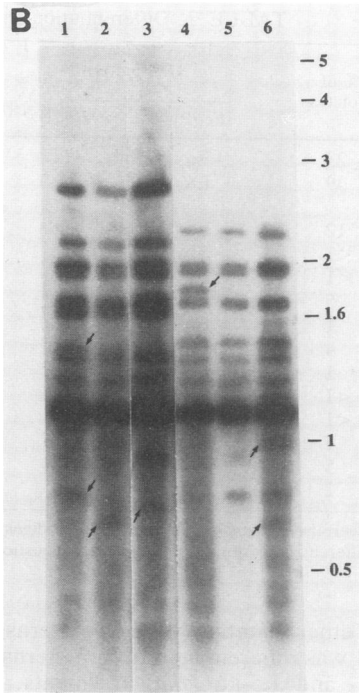


FIG. 4. DNA blot hybridization analysis of hardjo-bovis isolates. Samples of DNAs from hardjo-bovis isolates were digested with *Eco*RI (A) or *Hha*I (B), separated by electrophoresis in agarose gels, and transferred to nylon membranes. The DNA was hybridized with probe A (A) or probe B (B). The resulting autoradiograms are shown. (A) *Eco*RI patterns E1 through E10 are numbered at the top. Patterns E9 and E10 were separated on a different gel. The sizes (in kilobases) of molecular markers are shown on the left for lanes 1 to 8 and on the right for lanes 9 and 10. The 6- through 12-kb size markers migrated alike in both gels and are shown only on the left. (B) *Hha*I patterns H1 through H6 are numbered at the top. Arrows indicate distinguishing fragments. The sizes (in kilobases) of molecular markers are shown on the right.

Southern blot comparison of hardjo-bovis isolates. To select enzymes suitable for comparison of isolates by DNA hybridization, a preliminary study was done. DNA from a small number of isolates was digested with several enzymes (*Bgl*II, *Cla*I, *Eco*RI, *Hha*I, *Hin*dIII, *Nar*I, and *Xmn*I) and hybridized with probe A. The hybridization patterns of DNAs digested with *Eco*RI or *Hha*I resolved many more differences than those of DNAs digested with other enzymes (data not shown). The remaining hardjo-bovis isolates were compared by DNA blot hybridization using probe A to hybridize *Eco*RI-digested genomic DNA.

Ten different RFLP patterns (designated E1 through E10) were seen when *Eco*RI-digested DNA was hybridized with probe A (Fig. 4A). Type E1 isolates had a unique 3.1-kb *Eco*RI fragment. Type E2 isolates had a unique 3-kb *Eco*RI fragment and a 3.9-kb *Eco*RI fragment which was shared with patterns E3, E4, E5, and E7. E3 isolates had a unique 3.4-kb *Eco*RI fragment and a 5.3-kb *Eco*RI fragment shared with patterns E4 through E9. Type E4 was similar to E3 but lacked the E3 3.4-kb *Eco*RI fragment. E5, E6, E9, and E10 isolates lacked the 6-kb *Eco*RI fragment seen with other types. E6 isolates were similar to E5 isolates but lacked the 3.9-kb *Eco*RI fragment. Type E7 isolates were similar to E3 but had a unique 2.9-kb *Eco*RI fragment and lacked the E3 3.4-kb fragment. E7 and E8 isolates were similar, but type E8 lacked the 3.9-kb *Eco*RI fragment of E7. E9 isolates had a unique 4.8-kb *Eco*RI fragment. E10 isolates were similar to E1 but lacked the 3.1- and 6-kb *Eco*RI fragments.

Six different RFLP patterns (designated H1 through H6) were detected when *Hha*I-digested DNA was hybridized with probe B (Fig. 4B). Patterns H1 through H3 were more

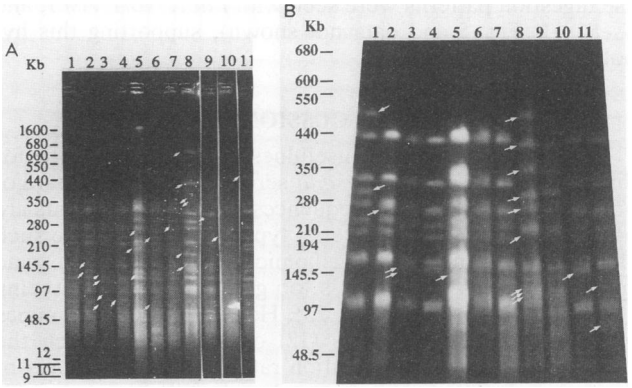


FIG. 5. Pulsed-field gel electrophoresis analysis of hardjo-bovis DNA. Genomic DNA prepared in agarose beads was digested with *Pac*I (A) or *Not*I (B) and separated through a 1% agarose gel at 200 V with pulse times increased from 1 to 50 s over 22 h (A) or 2 to 50 s over 24 h (B). Lanes contain DNAs from isolates as follows: lanes 1, 033; lanes 2, 045; lanes 3, 069; lanes 4, 057; lanes 5, 196; lanes 6, 145; lanes 7, 046; lanes 8, 067; lanes 9, 197; lanes 10, 323; lanes 11, 332. Arrows indicate distinguishing fragments.

TABLE 2. Differentiation and geographical distribution of genetic groups of hardjo-bovis

Genetic group	% of isolates	REA pattern		Hybridization pattern[a]		Geographical distribution
		*Eco*RI	*Hha*I	*Eco*RI	*Hha*I	
1	23	Ea	Ha	1	1	Africa, British Isles, North America, South America
2	39	Ea	Ha	2	1	Africa, British Isles, mainland Europe, North America, South America
3	12	Ea	Ha	3	1	South America
4	<1	Eb	Hd	4	2	Mainland Europe
5	<1	Ec	Ha	5	3	Mainland Europe
6	5	Ea	Ha	6	3	British Isles, mainland Europe
7	1	Ea	Ha	5	3	British Isles, mainland Europe
8[b]	4	Ea	Ha	5	3	North America
9	1	Ed	Ha	9	2	Mainland Europe
10	<1	Ed	Ha	10	2	Mainland Europe
11	<1	Ef	Ha	2	1	New Zealand
12	7	Ea	Hb	7	4	North America
13	5	Ea	Hb	8	5	North America
14	2	Ea	Hb	7	6	Middle East

[a] *Eco*RI digests were hybridized with probe A; *Hha*I digests were hybridized with probe B.
[b] Group 8 was differentiated from group 7 by *Pac*I digestion.

similar to each other than they were to patterns H4 through H6. Pattern H1 was differentiated from patterns H2 and H3 by unique 0.7- and 1.5-kb *Hha*I fragments. Pattern H2 differed from the H1 and H3 patterns by a unique 0.6-kb fragment. Pattern H3 was identified by using a unique 0.65-kb *Hha*I fragment. Patterns H4 through H6 were differentiated by unique *Hha*I fragments at 1.8 kb (H4), 0.6 kb (H5), and 1 kb (H6).

Hybridization and REA differences between isolates were used to group all hardjo-bovis isolates tested into 14 different groups (Table 2). The two human isolates fell into group 1, and all pig isolates fell into group 2. Since rRNA probes are useful for comparing different *Leptospira* serovars (10, 17), DNAs from representative isolates were also hybridized with 16S rRNA sequences. No differences in the 16S rRNA hybridization patterns were seen (data not shown).

Pulsed-field gel electrophoresis. Several isolates were analyzed by pulsed-field gel electrophoresis to determine whether the differences shown in Fig. 4 may be associated with genomic rearrangements. Several different endonuclease digestion patterns were seen with *Pac*I, *Not*I, *Asc*I, and *Sse*8387I (Fig. 5 and data not shown), supporting this hypothesis.

DISCUSSION

This report shows the usefulness of IS*1533* probes for differentiating *L. borgpetersenii* serovar hardjo type hardjo-bovis isolates. Repetitive sequences such as IS elements are not commonly used in bacterial typing schemes, since such elements can contribute to genomic instability (32). In some species, genomic plasticity is too great for IS-based typing schemes to be useful (7, 22, 23). However, if one examines only closely related strains (12) or species in which the transposition and recombination rates are low, members of the same clonal population can be identified by using IS-based probes (14, 20, 31). Previously, we showed that genetically similar leptospiral serovars had similar but distinct IS*1533* hybridization patterns (35). One conclusion derived from that study is that IS*1533* hybridization patterns change slowly within a given population (i.e., the presence of IS*1533* in the genome does not cause significant genome instability). Since IS*1533* is well distributed throughout the hardjo-bovis genome (Fig. 2), we hypothesized that IS*1533*-based probes would be sensitive markers of genetic change among hardjo-bovis isolates. By using these probes, 10 genetic groups of hardjo-bovis were identified, with four additional groups differentiated by REA (Table 2).

This study also extended the known geographical distribution of hardjo-bovis by identifying several hardjo-bovis isolates from Africa and South America. We believe that the different genetic groups detected with IS*1533* probes represent different clonal populations of hardjo-bovis, since specific groups were often localized to specific geographical regions. For example, 92% of the South American isolates were clustered in group 3, with the remaining isolates being typed as group 1 or 2. Isolates belonging to groups 8, 12, and 13 were found exclusively in North America. Several distinct genetic groups of hardjo-bovis from Europe were isolated (Table 2), and novel types from New Zealand and Israel were identified. Since members of groups 1 and 2 were isolated from most regions tested, these organisms may represent a common ancestral link between different genetic groups. Little genetic change was detected during in vitro culture of hardjo-bovis isolates, which shows that events leading to altered hybridization patterns are rare.

The genetic differences among hardjo-bovis groups can occur through several mechanisms. *Leptospira* serovars differ in the IS*1533* copy number (35), suggesting that IS*1533* transposes. Pulsed-field gel electrophoresis of large restriction fragments generated by rare cutting enzymes suggests that some of the genetic differences among hardjo-bovis isolates may result from genetic rearrangements. The hardjo-bovis genome contains at least two classes of repetitive sequences, IS*1533* (34) and a newly identified sequence of unknown function (16). Either of these elements could provide suitable targets for recombination. Since pulsed-field gel electrophoresis analysis of DNAs from several hardjo-bovis isolates showed the presence of several RFLPs, it appears that the hardjo-bovis genome may be less stable than the genome of *L. interrogans* serovar icterohaemorrhagiae (9). The IS*1533* probes described here have been useful for developing a better understanding of the epidemiology of hardjo-bovis infections. These probes may

help identify genetic differences associated with differences in pathogenicity.

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