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Pulsed-Field Gel Electrophoresis of *Not*I Digests of Leptospiral DNA: a New Rapid Method of Serovar Identification

J. L. HERRMANN, E. BELLENGER, P. PEROLAT, G. BARANTON, AND I. SAINT GIRONS*

Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, 75724 Paris Cedex 15, France

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Fingerprints for 72 reference serovar strains of pathogenic *Leptospira* spp. were obtained by pulsed-field gel electrophoresis (PFGE) following *Not*I restriction digests of the chromosome. These strains included the serovar reference strains of serogroups Australis, Ballum, Bataviae, Grippotyphosa, Panama, Pomona, and Pyrogenes. Sixty-four serovars could be identified by a unique *Not*I restriction profile. The remaining serovars were differentiated by chromosomal digestion with *Sgr*AI. These included four serovars from serogroup Australis, two serovars from serogroup Ballum, and two serovars from serogroup Bataviae. Thirteen of 18 recent clinical isolates identified by microagglutination test and cross-adsorption procedure were correctly typed by PFGE. The results indicate that PFGE, which is considerably more rapid than serology, should be useful for identification and epidemiological studies.

The order *Spirochaetales* comprises two families of thin, motile, spiral-shaped eubacteria, the *Spirochaetaceae* and the *Leptospiraceae* (5, 21). Within the family *Leptospiraceae*, pathogenic *Leptospira* spp. comprise six officially recognized genomic species (32): *Leptospira interrogans*, *L. weilii*, *L. borgpetersenii*, *L. inadai*, *L. noguchii*, and *L. santarosai*. A seventh group of pathogenic strains has been unofficially named *Leptospira alstoni* (13).

Pathogenic *Leptospira* spp. are divided into serovars by a serotypic classification based on a microagglutination test (MAT) used in a cross-adsorption procedure (9). Antigenically related serovars constitute serogroups which are composed of 203 serovars in 23 serogroups (14). The identification of each serovar is tedious, and the method requires the maintenance of a comprehensive collection of reference strains and their corresponding rabbit immune sera (7). The time required for the identification of unknown strains is at least 2 months, mainly because of the time needed for the preparation of immune serum in rabbits. This long interval does not facilitate epidemiological surveillance (9).

To explore the genetic basis of *Leptospira* classification and to facilitate the diagnosis of leptospirosis, studies have been performed by DNA-DNA hybridization (4, 10, 19, 32), DNA-rRNA hybridization (12, 20), and restriction endonuclease analysis (REA) (8, 16–18, 22–24, 26–28, 33, 34). The REA technique has allowed differentiation between closely related serovars. In this procedure, a large number of fragments are generated after restriction with an endonuclease such as *Eco*RI or *Hin*dIII. The resultant patterns are often smeared as the molecular sizes of the fragments decrease. Consequently, analysis of REA patterns is difficult and time-consuming. DNA hybridization using repetitive sequences (33, 34) or genomics probes (12, 15, 20, 25, 29–31), simplifies the interpretation and allows direct identification of specific serovars of *Leptospira* species from clinical samples. However, the method is still cumbersome even when nonradioactive probes are used (25).

* Corresponding author.

We have been using pulsed-field gel electrophoresis (PFGE) to analyze *Leptospira* DNA. Not only has it allowed us to determine its genome size (5,000 kbp) (2), but, by using the *Not*I restriction enzyme, we found that serovars of serogroup Icterohaemorrhagiae yielded unique fingerprint patterns (11). Moreover, these serovars show a strong genetic stability, since given serovars isolated from different regions of the world have been shown to have identical fingerprints (11). Here we extend our previous work to incorporate the other most frequently isolated serogroups in human and animal leptospirosis. With few exceptions, most strains could be identified by PFGE. Moreover, we show that the majority of recently isolated clinical strains can be readily identified by this method.

MATERIALS AND METHODS

Bacterial strains and media. A total of 72 *Leptospira* strains from the National Reference Centre Collection (Paris, France) were studied. These include all serovar reference strains of serogroups Australis, Ballum, Bataviae, Canicola, Grippotyphosa, Panama, Pomona, and Pyrogenes (Table 1). In addition, 18 recent clinical isolates (low-passage strains with less than 10 subcultures) from France, New Caledonia, Tahiti, Korea, Turkey, and Vietnam (isolated from blood, cerebrospinal fluid, and urine between November 1989 and April 1991) and belonging to serogroups Bataviae, Canicola, Grippotyphosa, Pomona, and Pyrogenes were analyzed (Table 2). Methods for serological identification (9) and growth of strains have been previously reported (11).

DNA extraction and digestion and PFGE. Except when noted in the text, PFGE experiments were performed as described previously (11). *Sgr*AI (CPu/CCGGPyG) was purchased from Boehringer. Most experiments made use of a Pharmacia-LKB apparatus (Uppsala, Sweden); where noted, a clamped homogenous field electrophoresis apparatus (CHEF DR-II; Bio-Rad Laboratories, Richmond, Calif.) was used. Multimers of the DNA from a 44.3-kbp bacteriophage λ derivative (6) and *Saccharomyces cerevisae* chromosomes (Bio-Rad) were used as standards.

TABLE 1. Serovar reference strains from pathogenic *Leptospira* spp

Serogroup	Serovar	Reference strain
Australis	australis[a]	Ballico
	bratislava	Jez-bratislava
	fugis	Fudge
	hawain	LT 62-68
	jalna	Jalna
	lora	Lora
	muenchen	München C 90
	nicaragua	1011
	peruviana	V 42
	ramisi	Musa
	rushan	507
	soteropolitana	R 93
	bajan	Toad 60
	pina	LT 932
Ballum	arborea	Arborea
	ballum[a]	Mus 127
	castellonis	Castellon 3
	kenya	Njenga
	peru	MW 10
Bataviae	argentiniensis	Pedulo
	balboa	735 U
	bataviae[a]	Swart
	brasiliensis	An 776
	claytoni	1348 U
	djatzi	HS 26
	kobbe	CZ 320
	paidjan	Paidjan
	rioja	M 12
	losbanos	LT 101-69
	santarosa	LT 21-74
Canicola	bafani	Bafani
	benjamini	Benjamin
	bindjei	Bindjei
	broomi	Patane
	canicola[a]	Hond Utrecht IV
	galtoni	LT 1014
	jonsis	Jones
	kamituga	Kamituga
	kuwait	136/2/2
	malaya	H 6
	portlandvere	MY 1039
	schueffneri	Vleermuis 90 C
	sumneri	Sumner
Grippotyphosa	canalzonae	CZ 188
	grippotyphosa[a]	Moskva V
	huanuco	M 4
	muelleri	RM 2
	ratnapura	Wumalasena
	valbuzzi	Valbuzzi
	vanderhoedeni	Kipod 179
Panama	cristobali	1996 K
	mangus	TRVL/CAREC 137774
	panama[a]	CZ 214
Pomona	kunming	K 5
	mozdok	5621
	pomona[a]	Pomona
	proechimys	1161 U
	tropica	CZ 299
	tsaratsovo	B 81/7
Pyrogenes	abramis	Abraham
	alexi	HS 616
	biggis	Biggs
	camlo	LT 64-67
	guaratuba	An 7705
	hamptoni	Hampton
	kwale	Julu
	manilae	LT 398
	myocastoris	LSU 1551
	princestown	TRVL 112499
	pyrogenes[a]	Salinem
	robinsoni	Robinson
	varela	1019
	zanoni	Zanoni

[a] So-called "leader" reference strain.

RESULTS

Fingerprints of serovars given by *Not*I restriction patterns. Rare-cutting endonucleases *Not*I and *Sgr*AI were used in this study, since they gave small numbers of restriction fragments. Sixty-four of 72 reference serovars yielded unique *Not*I fingerprints (Fig. 1A, 2, 3, 4A, 5A, 6A, 7A, and 7B). The pattern was reproducible, as each serovar had an identical pattern when tested at least five times. Two Australis serovars, soteropolitana and peruviana, differ by the presence of one band (220 kb) in serovar soteropolitana and a doublet (100 kb) in peruviana (Fig. 1A, lanes 8 and 9). In serogroup Bataviae, serovars claytoni and djatzi, differ by the presence of one band (460 kb) in serovar claytoni and a doublet (230 kb) in serovar djatzi (Fig. 3, lanes 5 and 6). In serogroup Pomona, serovars mozdok and tsaratsovo are difficult to distinguish by MAT (28), but their *Not*I restriction patterns differ by the presence of two bands (110 and 400 kbp) in serovar tsaratsovo and one band (460 kbp) in serovar mozdok (Fig. 6A, lanes 3 and 7).

A total of 8 of the 72 serovars could not be differentiated by *Not*I restriction analysis. These included (i) serovars lora, muenchen, jalna, and bratislava of serogroup Australis (Fig. 1A, lanes 3 to 6; these 4 serovars are very similar serologically and have similar REA patterns [8]); (ii) serovars castellonis and arborea of serogroup Ballum (Fig. 2, lanes 2 and 3); and (iii) serovars losbanos and bataviae of serogroup Bataviae (Fig. 3, lanes 11 and 12).

The use of *Sgr*AI allowed us to differentiate between these serovars. These serovars differ by the presence of one band (180 kb) in jalna and lora and one band (260 kb) in bratislava and muenchen (Fig. 1B, lanes 2, 3, 4, and 5). Serovars lora and jalna and serovars bratislava and muenchen were differentiated on the basis of a 325- to 350-kbp fragment present only in serovars lora and bratislava (Fig. 1B, lanes 2 and 5).

TABLE 2. Recent clinical isolates tested by PFGE

Serogroup (*n*)[a]	Serovar	Pathogenic origin	Geographical origin
Bataviae (2)	bataviae	ND[b]	Vietnam
Canicola (2)	canicola	ND	Korea
Canicola (1)	canicola	Humans	Tahiti
Grippotyphosa (1)	valbuzzi	Cattle	Turkey
Grippotyphosa (2)	grippotyphosa	Humans	France
Pomona (6)	pomona	Humans	New Caledonia
Pomona (1)	pomona	Humans	France
Pomona (1)	mozdok	Cattle	New Caledonia
Pyrogenes (2)	pyrogenes	Humans	New Caledonia

[a] Number of isolates.
[b] ND, not determined.

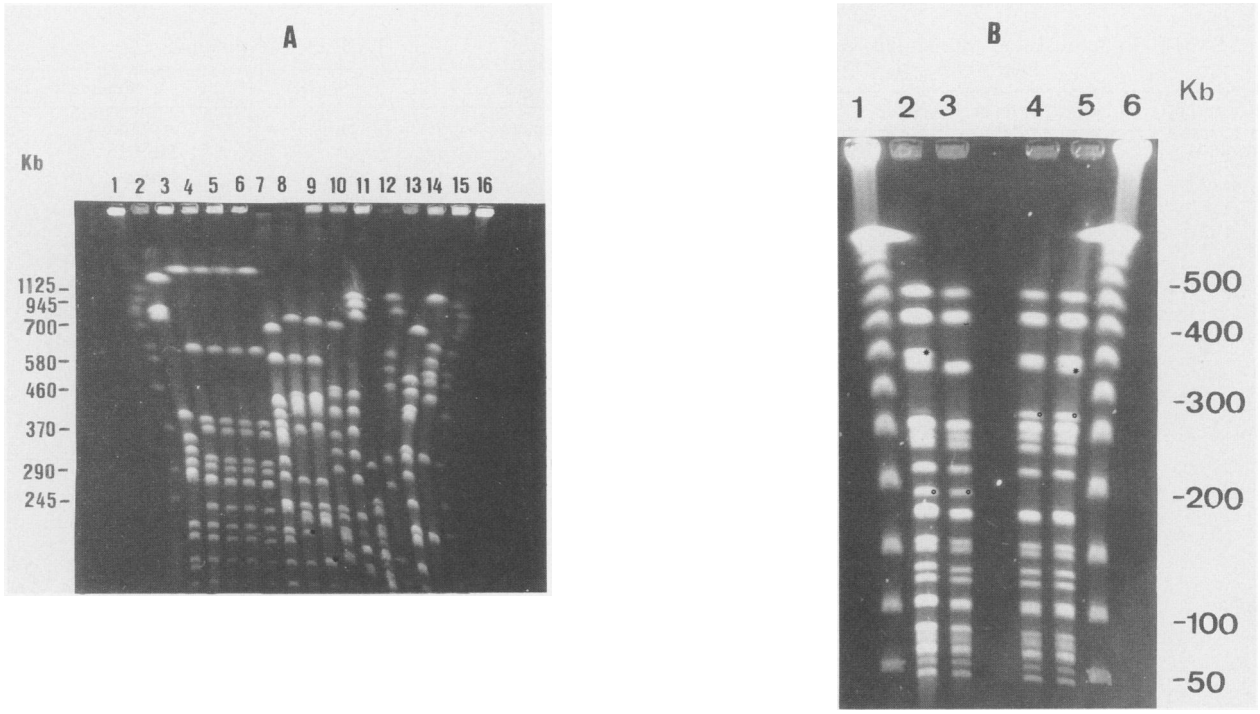

FIG. 1. PFGE of *Not*I (A) and *Sgr*AI (B) restriction fragments from serogroup Australis. (A) The digestion products were separated at 150 V for 40 h in 1% agarose–0.5× Tris-borate-EDTA. The three pulse times were 30 s over 13 h, 60 s over 13 h, and 120 s over 14 h. Asterisks indicate the specific bands for each serovar. Lanes: 1 and 16, size markers (*S. cerevisiae*); 2 to 15, serovars australis, lora, muenchen, jalna, bratislava, nicaragua, soteropolitana, peruviana, ramisi, fugis, rushan, bajan, hawain, and pina. (B) The digestion products were separated at 200 V for 24 h in 1% agarose–0.5× Tris-borate-EDTA. The three pulse times were 10 s over 9 h, 20 s over 8 h, and 30 s over 7 h. Small circles and asterisks indicate the specific bands for each serovar. Lanes: 1 and 6, size markers (λ); 2 to 5, serovars lora, jalna, muenchen, and bratislava.

A similar observation was made for serovars castellonis and arborea of serogroup Ballum (data not shown).

Identification of clinical isolates. Eighteen recent clinical isolates from humans and animals in different countries where leptospirosis is endemic were tested (Table 2). The serogroup of each strain was identified by the MAT technique and, in addition, the serovar was identified by a cross-adsorption test (9).

These clinical strains represented 29% of all the strains isolated or received in the laboratory for identification between November 1989 and April 1991. The remaining strains (a total of 45) belonged to serogroup Icterohaemorrhagiae serovar icterohaemorrhagiae; the results for these strains have been reported previously (11). The 29% of the total strains reported here are from serogroups Bataviae (2 of 18), Canicola (3 of 18), Grippotyphosa (3 of 18), Pomona (8 of 18), and Pyrogenes (2 of 18).

We compared the *Not*I restriction pattern of each clinical isolate with the *Not*I restriction patterns of all serovar reference strains belonging to the identified serogroup. This method allowed us to correctly identify 13 of the 18 clinical strains (Fig. 3, lanes 13 and 14; Fig. 4B, lanes 4 and 5; Fig. 5B, lanes 3 and 4; Fig. 6B, lanes 3 to 9). These 13 recent clinical isolates present a *Not*I restriction pattern identical to that of the "leader" serovar reference strain of the identified serogroup (Table 1, footnote *a*). Note that these strains were isolated from different geographic areas (Table 2). The results suggest that the genome of the highly passaged (>100 times) strains isolated in the laboratory many years ago are not markedly different from recent clinical isolates. Five isolates presented *Not*I restriction patterns which differ from those of any of the reference serovar strains. However,

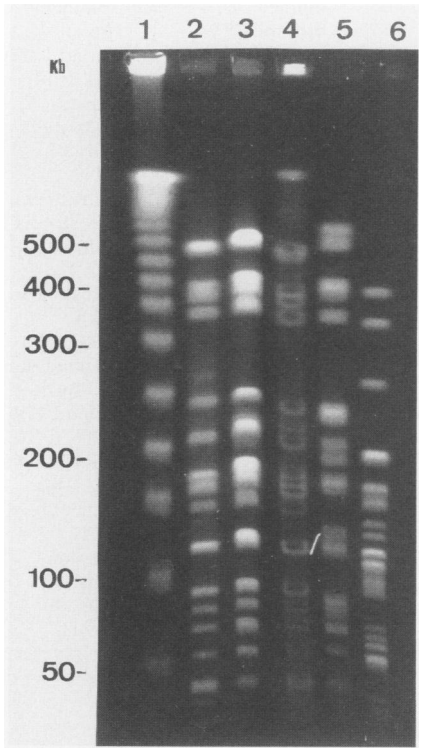

FIG. 2. PFGE of *Not*I restriction fragments from serogroup Ballum. The digestion products were separated at 200 V for 24 h in 1% agarose–0.5× Tris-borate-EDTA. The four pulse times were 5 s over 6 h, 15 s over 10 h, 30 s over 6 h, and 50 s over 2 h. Lanes: 1, size markers (λ); 2 to 6, serovars castellonis, arborea, ballum, kenya, and peru.

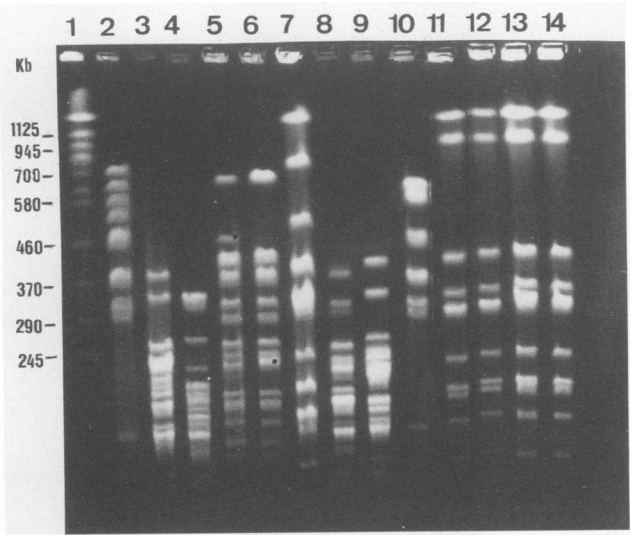

FIG. 3. PFGE of *Not*I restriction fragments from serogroup Bataviae. The digestion products were separated in 1% agarose–0.5× Tris-borate-EDTA with the following ramping program: 10 to 60 s over 20 h at 150 V followed by a second ramping of 60 to 240 s over 20 h at 100 V. Asterisks indicate the specific bands for each serovar. Lanes: 1, size markers (*S. cerevisiae*); 2 to 12, serovars argentiniensis, balboa, brasiliensis, claytoni, djatzi, kobbe, paidjan, rioja, santarosa, bataviae, and losbanos; 13 and 14, clinical strains.

these strains were identified by the MAT and cross-adsorption procedures as Canicola serovar canicola (Fig. 4B, lane 6), Grippotyphosa serovar valbuzzi (Fig. 5B, lane 2), Pomona serovar mozdok (Fig. 6C, lane 4), and Pyrogenes serovar pyrogenes (Fig. 7C, lanes 3 and 4).

DISCUSSION

We have used PFGE to determine the *Not*I (and in some cases the *Sgr*AI) digestion fingerprints for approximately half of the known serovars of pathogenic *Leptospira* spp. (reference 11 and this study). Our results indicate that 64 of 72 serovars can be identified by their *Not*I restriction patterns. The distinction between serovars by PFGE is significant, as it indicates that the serological system for serovar classification has a recognizable genetic basis. On the other hand, no correlation was observed between different serovars of a particular serogroup and their PFGE digestion fingerprints (reference 11 and this study). Similar conclusions have been suggested by Marshall et al. using REA (17). REA confirmed the hypothesis that serogroup classification has no biological significance apart from serological typing. In addition, few relationships were observed between DNA-DNA homology and PFGE pattern. We have shown that serovars, within a given genomic species, have different PFGE fingerprints. Strains could have evolved differently

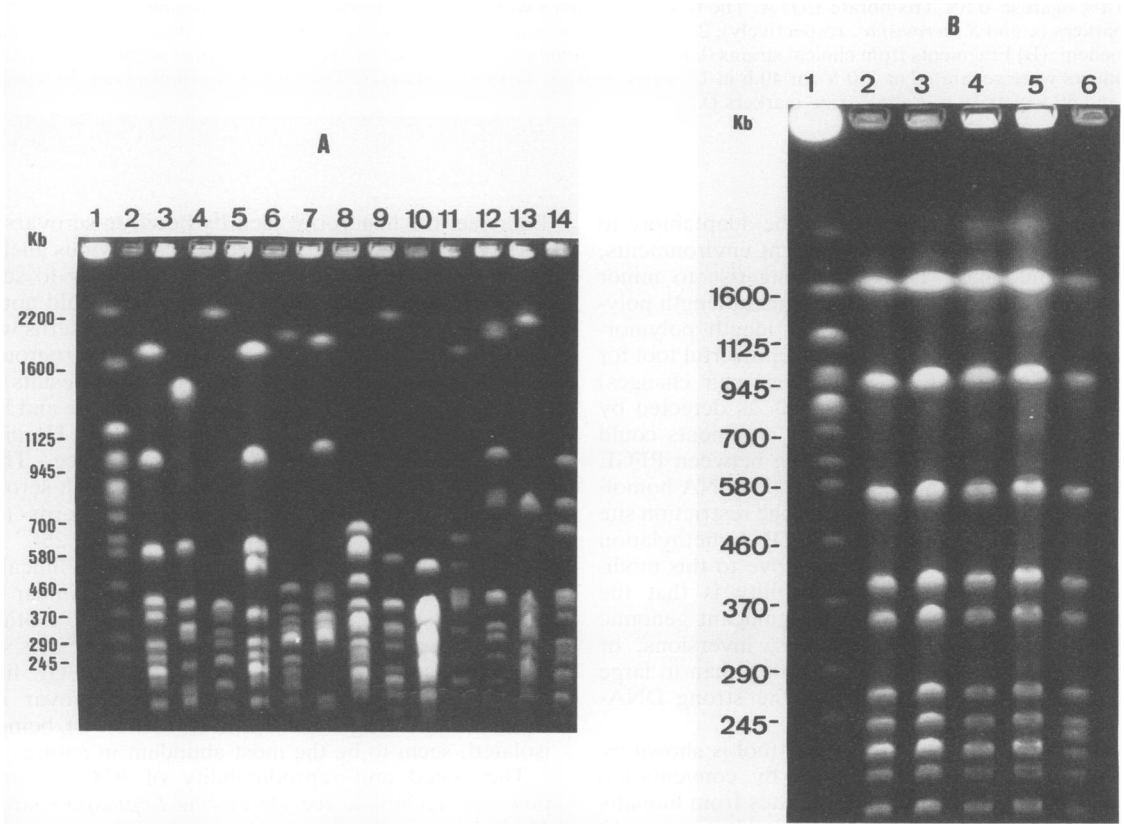

FIG. 4. PFGE of *Not*I restriction fragments. (A) Fragments from serogroup Canicola. The digestion products were separated at 150 V for 40 h in 1% agarose–0.5× Tris-borate-EDTA by using two ramping programs: 30 to 60 s over 20 h and then 60 to 240 s over 20 h. Lanes: 1, size markers (*S. cerevisiae*); 2 to 14, serovars canicola, bindjei, malaya, portlandvere, sumneri, jonsis, bafani, benjamini, galtoni, schueffneri, broomi, kuwait, and kamituga. (B) Fragments from clinical strains (lanes 4 to 6) compared with those of reference serovar canicola (lane 2) and a highly passaged clinical strain isolated in France (lane 3) and identified by MAT as serovar canicola. The digestion products were separated at 150 V for 42 h in 1% agarose–0.5× Tris-borate-EDTA. The four pulse times were 30 s over 12 h, 60 s over 10 h, 120 s over 10 h, and 240 s over 10 h. Lane 1: size markers (*S. cerevisiae*).

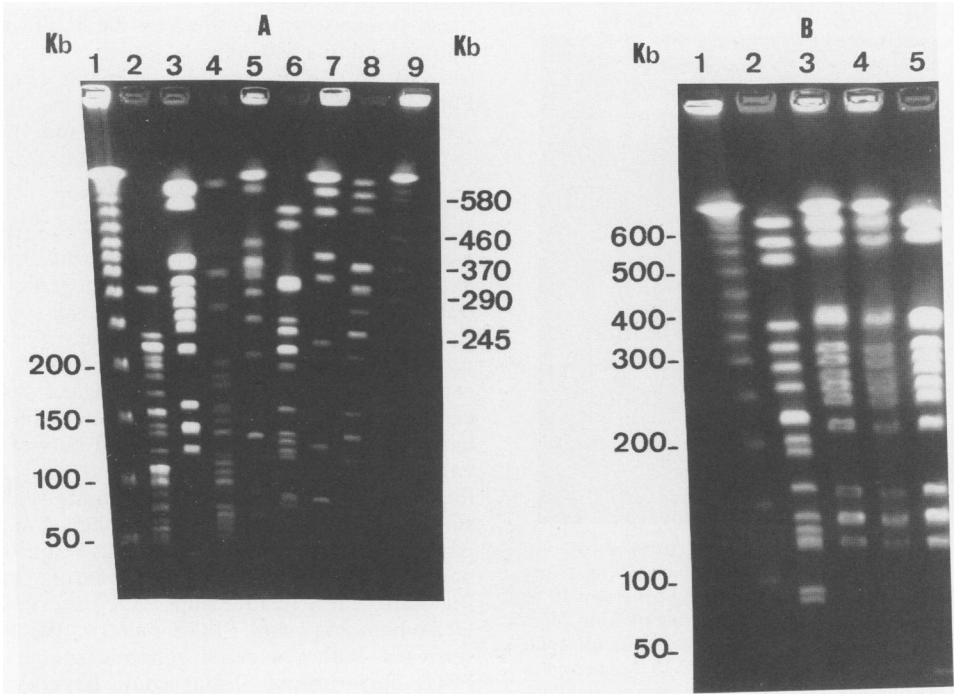

FIG. 5. PFGE of *Not*I restriction fragments. (A) Fragments from serogroup Grippotyphosa. The digestion products were separated at 150 V for 40 h in 1% agarose–0.5× Tris-borate-EDTA. The three pulse times were 10 s over 12 h, 20 s over 12 h, and 50 s over 16 h. Lanes: 1 and 9, size markers (λ and *S. cerevisiae*, respectively); 2 to 8, serovars canalzonae, grippotyphosa, huanuco, muelleri, ratnapura, valbuzzi, and vanderhoedeni. (B) Fragments from clinical strains (lanes 2 to 4) compared with those from reference serovar grippotyphosa (lane 5). The digestion products were separated at 150 V for 40 h in 1% agarose–0.5× Tris-borate-EDTA. The three pulse times were 10 s over 12 h, 20 s over 12 h, and 50 s over 16 h. Lane 1: size markers (λ).

from a common ancestor, depending on the adaptability to different ecological niches. In such different environments, bacterial DNA could have evolved, giving rise to minor changes detectable only by restriction fragment length polymorphism analysis. Restriction fragment length polymorphism analysis was introduced in 1980 as a powerful tool for detecting small differences (i.e., single base pair changes) among DNAs of closely related organisms as detected by conventional genomic analysis (3). Other arguments could be given to explain the lack of correlation between PFGE and the definition of a species based on DNA-DNA homology. The simplest explanation may be that the restriction site distributions are related to differences in DNA methylation (1); however, *Not*I seems to be less sensitive to this modification (1). The most interesting possibility is that the restriction site redistribution is due to significant genomic rearrangements, mainly by translocations, inversions, or distribution of repetitive elements (33), rather than to large insertions or deletions, as indicated by the strong DNA-DNA homology.

The power of PFGE as an identification tool is shown by the consistency of the results obtained by conventional methods and PFGE for the 63 clinical isolates from humans or animals obtained from endemic countries (reference 11 and this study). Note that eight serovars of serogroups Australis, Ballum, and Bataviae with similar *Not*I restriction patterns could be distinguished only by using *Sgr*AI. These results were in agreement with REA (8) and DNA-rRNA hybridization results for serogroup Australis but were first demonstrated here for serogroup Ballum. For serogroup Bataviae, the fingerprint identity between serovars bataviae and losbanos is well correlated with a previous analysis (14).

The five recent clinical isolates belonging to serogroups Grippotyphosa, Pomona, and Pyrogenes could not be identified by comparing their *Not*I restriction patterns with those of all the reference serovars within a serogroup. Their identification according to serovar (see Results) showed divergence between the serological technique and PFGE. In addition, the two strains from serovar hardjo, Hardjoprajitno and Hardjo-Bovis, also showed divergence. These two strains, which are very difficult to distinguish serologically, have, however, very distinct PFGE patterns (data not shown), in agreement with REA results (22, 26).

We observed that 13 of the 18 recent clinical isolates tested possessed a *Not*I restriction pattern similar to that of the leader serovar reference strain (Table 1, footnote *a*) of the serogroup to which they belonged. The results suggested that most serovars do not vary in their PFGE fingerprint regardless of their origin. The leader serovar reference strains of the serogroups (Table 1, footnote *a*), being the first isolated, seem to be the most abundant in nature.

The speed and reproducibility of PFGE represents a powerful technique for identifying *Leptospira* strains. The *Not*I restriction profiles for 100 of the 203 reference serovars are known (reference 11 and this study). We have been able to identify 92% (58 of 63) of recent clinical isolates received in the laboratory within 6 days after subculture (reference 11 and this study). This identification method is presently performed routinely in our laboratory. We first identify the serogroup of the clinical isolate by MAT, and concomitantly

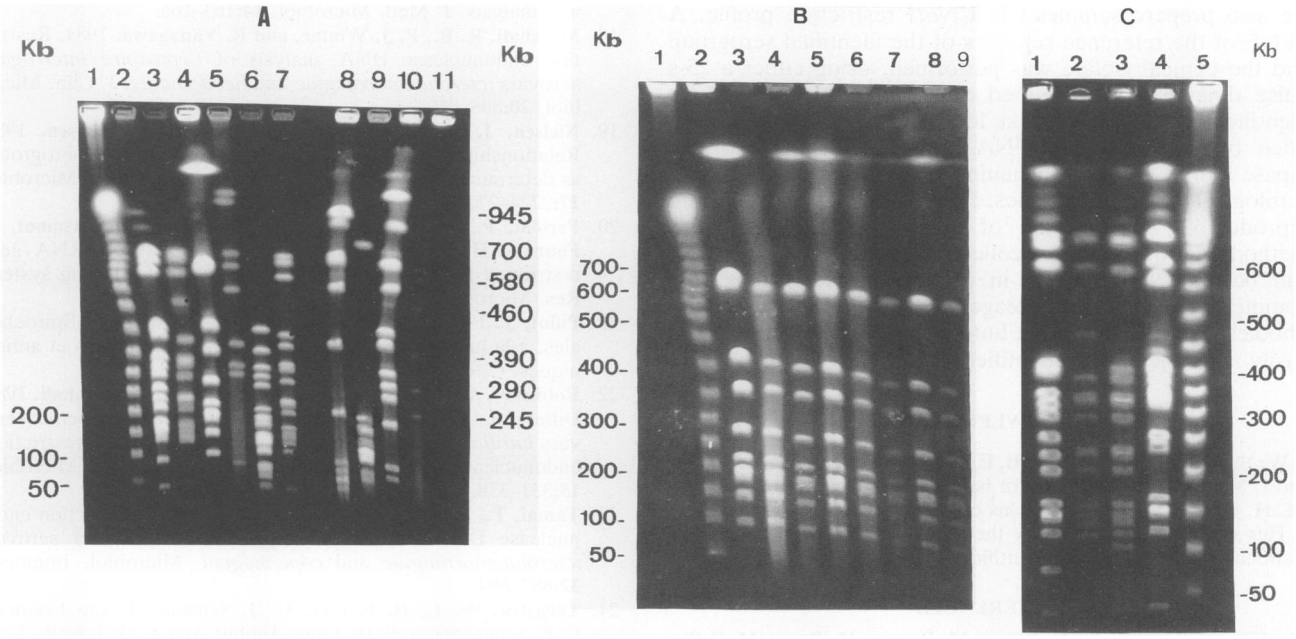

FIG. 6. PFGE of *Not*I restriction fragments. (A) Fragments from serogroup Pomona (lanes 2 to 7) and Panama (lanes 8 to 10). The digestion products were separated at 200 V for 24 h in 1% agarose–0.5× Tris-borate-EDTA. The three pulse times were 25 s over 10 h, 50 s over 8 h, and 100 s over 6 h. Asterisks indicate the specific bands for each serovar. Lanes: 1 and 11, size markers (λ and *S. cerevisiae*, respectively); 2 to 7, serovars kunming, mozdok, pomona, proechimys, tropica, and tsaratsovo; 8 to 10, serovars cristobali, mangus, and panama. (B) Fragments from clinical strains (lanes 3 to 9) compared with those from reference serovar pomona (lane 2). The digestion products were separated at 200 V for 24 h in 1% agarose–0.5× Tris-borate-EDTA. The three pulse times were 25 s over 10 h, 50 s over 8 h, and 100 s over 6 h. Lane 1: size markers (λ). (C) Fragments from one clinical strain (lane 4) compared with those from reference serovars kunming, mozdok, and tsaratsovo (lanes 1 to 3). The digestion products were separated at 200 V for 24 h in 1% agarose–0.5× Tris-borate-EDTA. The three pulse times were 10 s over 5 h, 30 s over 12 h, and 60 s over 7 h. Lane 5: size markers (λ).

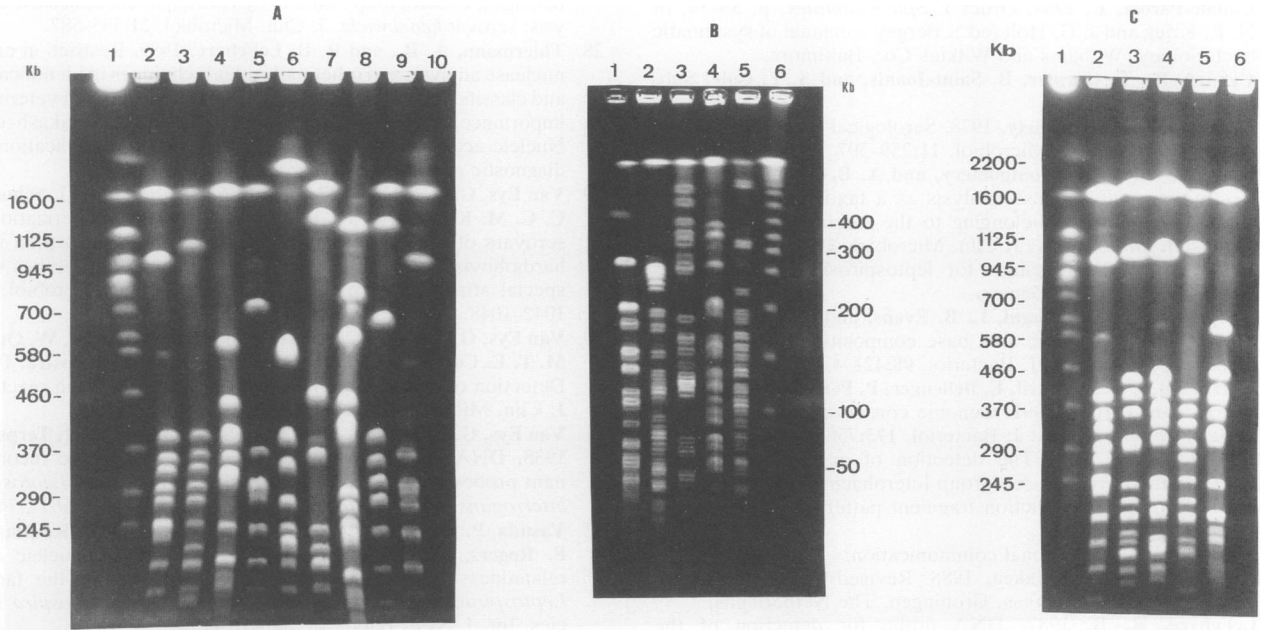

FIG. 7. PFGE of *Not*I restriction fragments. (A) Fragments from serogroup Pyrogenes. The digestion products were separated at 150 V for 40 h in 1% agarose–0.5× Tris-borate-EDTA. The three pulse times were 30 s over 17 h, 60 s over 13 h, and 150 s over 10 h. Lanes: 1, size markers (*S. cerevisiae*); 2 to 10, serovars pyrogenes, abramis, biggis, camlo, guaratuba, manilae, myocastoris, robinsoni, and zanoni. (B) Fragments from serogroup Pyrogenes. The digestion products were separated at 200 V for 24 h in 1% agarose–0.5× Tris-borate-EDTA. The three pulse times were 5 s over 7 h, 10 s over 10 h, and 25 s over 7 h. Lanes: 1 to 5, serovars alexi, hamptoni, kwale, princestown, and varela; 6, size markers (λ). (C) Fragments from clinical strains (lanes 3 and 4) compared with those from reference serovars pyrogenes, abramis, and biggis (lanes 2, 5, and 6, respectively). Lane 1: size markers (*S. cerevisiae*).

we also prepare samples for a *Not*I restriction profile. A PFGE of the reference serovars of the identified serogroup and the clinical isolate was performed, using either a 25-s pulse time or a programmed run. The 8% of strains not identified by PFGE may take longer to be completely identified by genetic (DNA-DNA hybridization), phenotypic (lipase activity and 8-azaguanine resistance, etc.) (32), and serological (MAT) techniques. PFGE provides a reliable and reproducible identification of *Leptospira* serovars. This method does not require a collection of microorganisms and can be achieved rapidly, in contrast to serotyping. No commercially unavailable reagents (such as monoclonal antibodies) are required. Last but not least, the results can be easily and objectively quantified, in contrast to REA.

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