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Characterization of *Leptospira* Isolates from Serovar Hardjo by Ribotyping, Arbitrarily Primed PCR, and Mapped Restriction Site Polymorphisms

PHILIPPE PEROLAT,[1]* FABRICE MERIEN,[1] WILLIAM A. ELLIS,[2] AND GUY BARANTON[3]

Laboratoire des Leptospires, Institut Pasteur, Nouméa, New Caledonia[1]; Veterinary Sciences Division, Stormont, Belfast BT4 3SD, Northern Ireland, United Kingdom[2]; and Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, 75724 Paris Cedex 15, France[3]

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***Leptospira* serovar hardjo isolates of the hardjoprajitno and hardjobovis genotypes were characterized by ribotyping, arbitrarily primed PCR (AP-PCR) fingerprinting, and the study of mapped restriction site polymorphisms (MRSPs) in *rrs* and *rrl* genes. After restriction of chromosomal DNA with *Bgl*II, *Eco*RI, or *Hin*dIII, each genotype was individualized with a distinct ribotype. The fingerprints produced by AP-PCR with seven primers clearly separated the two groups; primers KF and RSP produced species-specific products which assigned hardjoprajitno and hardjobovis isolates to the species *L. interrogans* sensu stricto and *L. borgpetersenii*, respectively. Furthermore, AP-PCR fingerprints gave evidence of a considerable genomic heterogeneity at the strain level among the hardjobovis group. Conversely, the hardjoprajitno group was homogeneous. MRSP profiles in ribosomal genes indicated that hardjoprajitno and hardjobovis isolates belonged to *L. interrogans* MRSP group B and *L. borgpetersenii* group C, respectively. AP-PCR and determination of MRSPs in ribosomal genes proved to be quick and reliable methods for typing *Leptospira* strains and for studying intraspecific population structures.**

Leptospirosis, which is caused by *Leptospira interrogans*, is a zooanthroponosis which has a worldwide distribution. For taxonomic purposes and as an aid to epidemiological studies, the parasitic leptospires have been subdivided into serovars by their agglutination-absorption patterns (18). Antigenically related serovars constitute serogroups. Twenty-three serogroups are recognized, and these 23 serogroups contain 202 serovars (19). Alternative serological typing systems based on either monoclonal antibodies or factor analysis give results comparable to those of conventional serotyping methods (3, 18).

The advent of genetic typing methods has thrown the conventional serology-based taxonomy of the genus into some confusion, but these methods have provided rapid, reproducible typing protocols. Seven genospecies of parasitic leptospires have been described on the basis of DNA relatedness (35) and guanine-plus-cytosine (G+C) content studies (27, 39). The value of this system with respect to epidemiology is limited. Other genetic typing methods have proved to be of value and serve as supplementary or alternative typing systems, particularly in epidemiological studies, since they identify strain differences at the subserovar level. These include restriction enzyme analysis (REA) of chromosomal DNA by fixed-field gel electrophoresis (6, 22, 31, 32) or pulsed-field gel electrophoresis (PFGE) (15, 16), the use of recombinant DNA probes (20, 30, 33, 41), and ribotyping (24, 25).

Cattle act as maintenance hosts for serovar hardjo, which is endemic in the cattle populations of most countries. Its reported involvement in clinical disease varies. In the United Kingdom it is recognized as a cause of a distinctive acute agalactia in cattle and as a major factor in bovine abortion (7, 8). The other end of the spectrum is seen in New Zealand (4), where it is not recognized as a cause of clinical disease in cattle. While differences in cattle management practices and resulting herd immunity probably contribute to the different clinical situations observed in different countries and even between regions of countries, it is also probable that strain differences make an important contribution to these differences. The genomic heterogeneity of strains grouped as serovar hardjo was first demonstrated by using REA (23, 29), leading to the delineation of two subtypes, hardjoprajitno and hardjobovis. Subsequently, these two genotypes were also differentiated on the basis of lipopolysaccharide composition (34), ribosomal structure (17), percent G+C content (21), and antigenic structure (21) and with recombinant probes (20, 33, 40, 41). Within both genotypes, hardjobovis and hardjoprajitno strain variations may be detected by (i) REA (9, 10, 42) and (ii) probing with a repetitive sequence probe (42).

Despite good sensitivity and reproducibility, the current genotypic methods used for typing hardjo strains have some disadvantages. REA differentiation is based only on the high-molecular-weight fragments, since smearing occurs as the molecular size decreases. DNA hybridization with repetitive sequences (41, 42) or serovar-specific fragments (20, 33) simplifies the interpretation, but the method is still cumbersome. However, PFGE of restricted chromosomal DNA proved to be very useful for the identification of leptospires at the serovar level (15, 16) but still requires appreciable amounts of DNA.

PCR-based characterization methods use much less DNA, which is an important consideration when studying such slowly growing bacteria as leptospires. Arbitrarily primed PCR (AP-PCR) (36, 38) generates fingerprints that can be used to compare microorganisms at the species level (37) and within a species (36). This technique has recently been applied to the study of selected *Leptospira* strains (2, 26). A new PCR strategy, which is based on the study of mapped restriction site polymorphisms (MRSPs) in PCR-amplified *rrs* and *rrl* eubacterial ribosomal genes, was developed and tested on *Leptospira*

* Corresponding author. Mailing address: Laboratoire des Leptospires, Institut Pasteur, P.O. Box 61, Nouméa, 98845, New Caledonia (South Pacific). Phone: 687 272666. Fax: 687 273390.

TABLE 1. *Leptospira* field isolates of serovar hardjo used in the present study

Genotype and lane no.[a]	Strain	Country of origin[b]	Organ isolated from:	Species isolated from:	Source	Year of isolation	RSP group[c]
Hardjobovis							
1.	83-561(181)	N. Ireland	Oviduct	Bovine	W. Ellis	1983	−
2.	83-468(6105)	N. Ireland	Urine	Bovine	W. Ellis	1983	−
3.	Kidney 34	N. Ireland	Kidney	Bovine	W. Ellis	1979	−
4.	84-2793	N. Ireland	Kidney	Bovine	W. Ellis	1984	+
5.	83/615	N. Ireland	Milk	Bovine	W. Ellis	1983	−
6.	Ab56	N. Ireland	Fetus	Bovine	W. Ellis	1975	−
7.	82/1215	N. Ireland	Fetus	Bovine	W. Ellis	1982	−
8.	83-835	N. Ireland	Liver	Sheep	W. Ellis	1983	−
9.	LC80/22	England	Milk	Bovine	D. Pritchard	1980	−
10.	LC81/45F1	England	Fetus	Bovine	D. Pritchard	1981	+
11.	Michna 51	Scotland	Kidney	Bovine	S. Michna	1967	−
12.	N° 33	Germany	Urine	Bovine	S. Brem	1986	+
13.	N° 34	Germany	Urine	Bovine	S. Brem	1986	−
14.	N51	Switzerland	Kidney	Bovine	L. Corboz	1983	+
15.	N233	Switzerland	Kidney	Bovine	L. Corboz	1983	+
16.	6B	Portugal	Kidney	Bovine	M. Pereira	1987	+
17.	7B	Portugal	Kidney	Bovine	M. Pereira	1987	+
18.	LT371	Malaysia	NA[d]	Human	N. Stallman	NA	+
19.	LT428	Australia	NA	Bovine	N. Stallman	NA	−
20.	LT435	Australia	NA	Bovine	N. Stallman	NA	−
21.	S715	Italy	Urine	Bovine	Andreani	1967	−
22.	S731	Italy	Urine	Bovine	Andreani	1967	+
23.	84/2969	U.S.A.	Uterus	Bovine	W. Ellis	1984	+
24.	84/2894	U.S.A.	Kidney	Bovine	W. Ellis	1984	+
25.	Hb15(003)	U.S.A.	Kidney	Bovine	A. Thiermann	NA	−
26.	Anthony 1	Nigeria	Kidney	Bovine	A. Ezeh	NA	−
27.	SBF 31	Zimbabwe	Kidney	Bovine	S. Feresu	NA	+
Hardjoprajitno							
28.	79-712	N. Ireland	Fetus	Bovine	W. Ellis	1979	
29.	81-1665	N. Ireland	Fetus	Bovine	W. Ellis	1981	
30.	79-857	N. Ireland	Milk	Bovine	W. Ellis	1979	
31.	N2033	N. Ireland	Fetus	Bovine	W. Ellis	NA	
32.	N2004	N. Ireland	Oviduct	Bovine	W. Ellis	NA	
33.	80-270	N. Ireland	Kidney	Bovine	W. Ellis	1980	
34.	82-2316	N. Ireland	Fetus	Sheep	W. Ellis	1982	
35.	83-287	N. Ireland	Blood	Sheep	W. Ellis	1983	
36.	83-424	N. Ireland	Milk	Sheep	W. Ellis	1983	
37.	80-564	N. Ireland	Kidney	Horse	W. Ellis	1983	
38.	204	Scotland	Kidney	Bovine	S. Michna	1969	
39.	Anthony 7	Nigeria	NA	Human	A. Ezeh	NA	

[a] Lane numbers in Fig. 6A and B.
[b] N. Ireland, Northern Ireland; U.S.A., United States.
[c] Presence (+) or absence (−) of the 1,400-bp RSP-derived AP-PCR product in hardjobovis strains.
[d] NA, not available.

strains (26). MRSPs allowed the grouping of reference strains into 16 profiles, which subdivided the genomic species defined in DNA relatedness studies (27, 39).

The purpose of the study described here was to evaluate ribotyping, AP-PCR, and MRSPs in PCR-amplified rRNA genes as methods for characterizing and differentiating strains within serovar hardjo.

MATERIALS AND METHODS

Bacterial strains. Thirty-nine isolates from serovar hardjo, maintained at the Veterinary Sciences Division, Stormont, Belfast, United Kingdom, were studied. These strains, listed in Table 1, were previously typed by REA; 27 were hardjobovis and 12 were hardjoprajitno. In addition, the reference strain of serovar hardjo (strain Hardjoprajitno from *L. interrogans* [39]) and strain Sponselee (genotype hardjobovis from *L. borgpetersenii* [25, 26]) were included in the study. They were provided by the French National Reference Center Collection.

The hardjobovis isolates originated in various geographical areas: 20 from Europe (Northern Ireland, $n = 8$; England, $n = 2$; Germany, $n = 2$; Italy, $n = 2$; Portugal, $n = 2$; Switzerland, $n = 2$; The Netherlands, $n = 1$; Scotland, $n = 1$), 3 from the United States, 2 from Africa (Nigeria, $n = 1$; Zimbabwe, $n = 1$), 2 from Australia, and 1 from Malaysia. The hardjoprajitno isolates were all from Europe (Northern Ireland, $n = 10$; Scotland, $n = 1$), with the exception of one strain isolated in Nigeria.

In each procedure, the reference strains of *L. interrogans* (strain RGA) and *L. borgpetersenii* (strain Veldrat Batavia 46) were included as internal controls.

Culture conditions and preparation of DNA. Each strain was grown in EMJH medium (5) at 30°C with shaking until the stationary phase of growth was reached. DNA was extracted and purified as described by Brenner et al. (1).

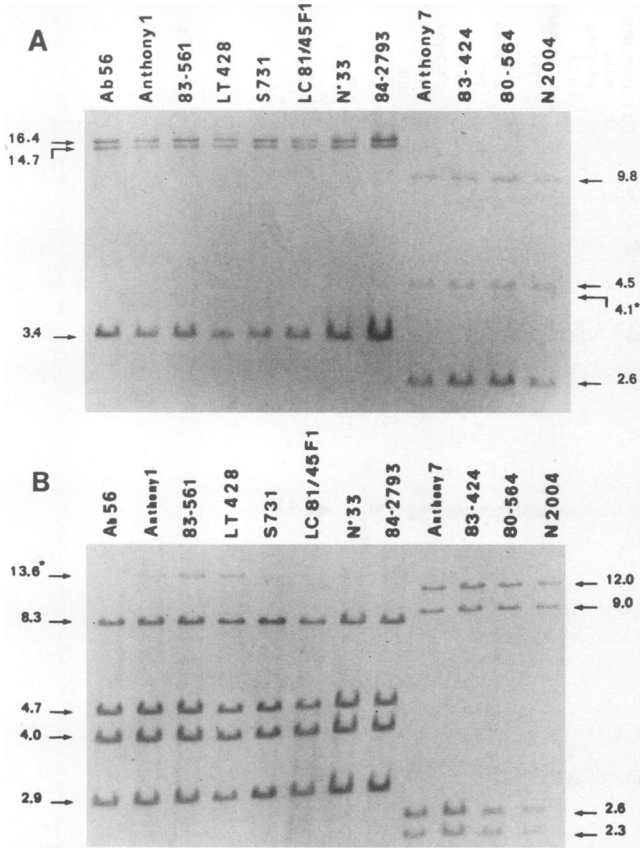


FIG. 1. Ribotypes of selected hardjobovis (first eight lanes) and hardjoprajitno (last four lanes) strains. DNAs were digested with *Bgl*II (A) and *Hin*dIII (B). *, weaker bands.

Ribotyping. Ribosomal DNA gene restriction patterns were obtained as described by Grimont and Grimont (14). Purified leptospiral DNA (5 μg) was mixed with 20 U of restriction enzyme in a 20-μl reaction mixture containing the manufacturer's buffer (Boehringer, Mannheim, Germany), and the mixture was incubated for 2 h at 37°C. According to the results of previous studies (6, 24, 25), *Bgl*II, *Eco*RI, and *Hin*dIII were used because they were useful in generating polymorphisms in leptospiral DNA. Digested DNA was separated by electrophoresis on a 0.8% agarose gel overnight at 2 V/cm in Tris acetate-EDTA buffer and was transferred onto Hybond C Extra filters (Amersham International, Amersham, England) by using the Vacugene system (Pharmacia LKB Biotechnology, Uppsala, Sweden). Southern blots of the DNA digests were hybridized with acetylaminofluorene-labelled 16S+23S rRNA from *Escherichia coli* (Eurogentec, Liège, Belgium) as described elsewhere (13) and according to the manufacturer's instructions.

AP-PCR. AP-PCR fingerprints were obtained as described previously (36, 37). Primers KF (5′-CAC GCA CAC GCA CAG AGA-3′), KG (5′-CAC ACG CAC ACG GAA GAA-3′), KN (5′-CCT TGC GCG CAT GTA CAT GG-3′), KpnR (5′-CCA AGT CGA CAT GGC ACR TGT ATA CAT AYG TAA C-3′), KZ (5′-CCC ATG TGT ACG CGT GTG GG-3′), RSP (5′-GGA AAC AGC TAT GAC CAT GA-3′), and SP (5′-TTG TAA AAC GAC GGC CAG-3′) were purchased from Genset (Paris, France). Fifty-microliter reaction mixtures were prepared with 100 ng of DNA–1× *Taq* polymerase buffer (100 mM Tris [pH 8.3 at 20°C], 500 mM KCl)–4 mM $MgCl_2$–0.2 mM (each) deoxynucleoside triphosphate–1 μM single nucleotide primer–5 μCi of [^{32}P]dCTP (3,000 Ci/mmol; Amersham)–1.25 U of *Taq* polymerase (Amersham). The reactions were cycled in a GeneAmp 9600 (Perkin-Elmer) 2 times through a low-stringency temperature profile (94°C for 5 min for denaturation, 40°C for 5 min for low-stringency annealing, and 72°C for 5 min for extension) and then 40 times through a high-stringency temperature profile (94°C for 1 min for denaturation, 60°C for 1 min for high-stringency annealing, and 72°C for extension). Five microliters of each reaction mixture was combined with 15 μl of 98% formamide dye, and this mixture was heated to 68°C for 15 min; 3 μl of each sample was loaded onto a 4% acrylamide–50% urea sequencing gel with 1× TBE (90 mM Tris-borate, 2 mM EDTA), and electrophoresis was done at 400 V overnight until the xylene cyanol tracking dye was approximately 10 cm from the bottom. pBR322 DNA digested with *Bst*NI (New England, Biolabs) and pBR328 digested with *Bgl*I-*Hin*fI (Boerhinger) were used

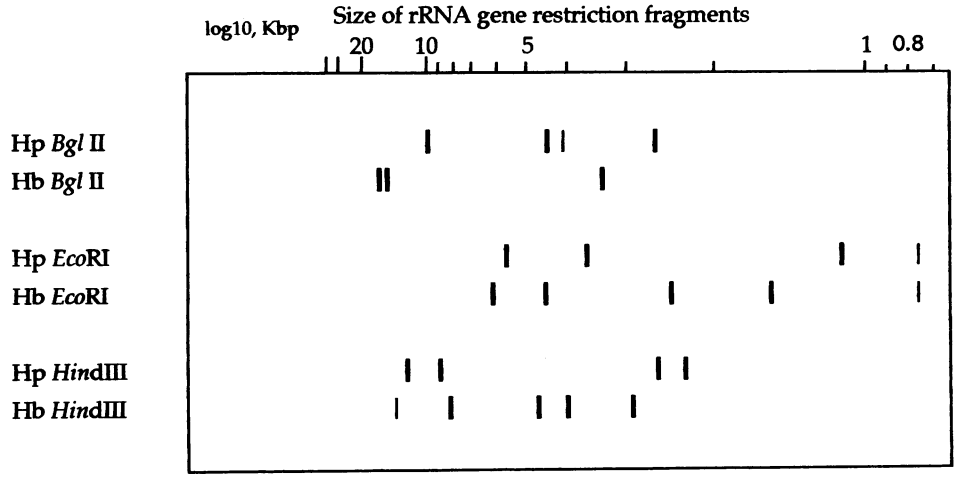
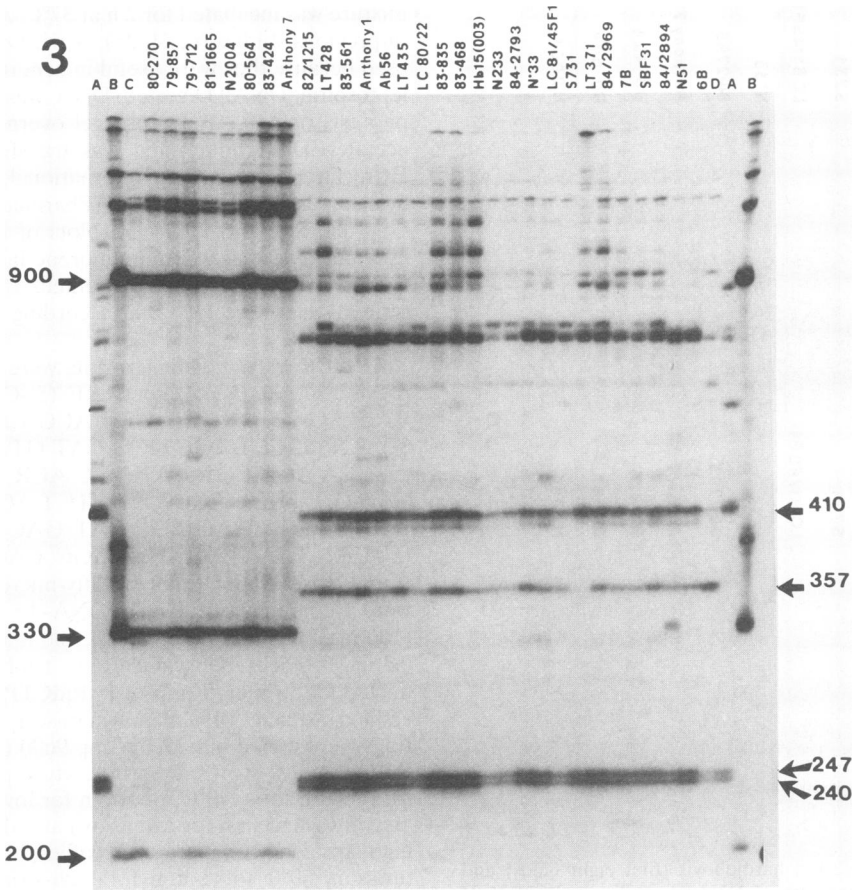


FIG. 2. Normalized graph of hardjoprajitno (Hp) and hardjobovis (Hb) ribotypes produced after *Bgl*II, *Eco*RI, or *Hin*dIII restriction. Thinner lines indicate weaker bands.



as molecular size markers. The gel was autoradiographed for 24 to 48 h on Kodak X-Omat X-ray film. For first analysis, AP-PCR products were analyzed on a 2% agarose gel (NuSieve 3:1; FMC, Rockland, Maine) with 0.5× TBE (3 V/cm during 16 h) and stained with ethidium bromide.

MPSPs in PCR-amplified *rrs* and *rrl* genes. Analysis of MRSPs in PCR-amplified *rrs* and *rrl* genes was performed as described by Ralph et al. (26). Primers developed by aligning the 16S and 23S sequences available for the members of the family *Leptospiraceae* with the eubacterial *rrs* and *rrl* genes in highly conserved regions, which matched at 90% with almost all such genes, were used. The following oligonucleotides were purchased from Genset (Paris, France): for the *rrs* gene, 16S-11 (5′-GGC TGC AGT CGA CGT TTG ATC CTG GCT CAG-3′) and 16S-1507 (5′-CCA GAT CTG AGC TCA AGG AGG TGA TCC AGC-3′); for the *rrl* gene, 23S-220 (5′-AAC CAG AAT TCC GTC AGT AGC GGT GAG CGA A-3′) and 23S-1432 (5′-GGT GTC GAC TAT GAA CCT GCT TCC CAT CGA CTA C-3′).

PCR was performed with approximately 5 ng of genomic DNA. For each strain, 10^6 cells were washed in sterile distilled water and were collected by centrifugation at 13,000 × *g* for 15 min. The pellets were resuspended in 10 μl of TE buffer and were heated at 96°C during 10 min. Amplification of DNA was performed in a total volume of 50 μl with 1× *Taq* polymerase buffer–1.75 mM $MgCl_2$–0.2 mM (each) deoxynucleoside triphosphate–500 nM primer–2.5 μCi of [^{32}P]dCTP (3,000 Ci/mmol)–1.25 U of *Taq* polymerase. Cycling was done as 30 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C.

Restriction enzyme digestions were performed with *Dde*I, *Hha*I, *Nla*IV, and *Taq*I for the *rrs* gene. *Hha*I, *Hph*I, and *Mnl*I were used for the *rrl* gene. Four microliters of PCR product was cleaved with 5 to 10 U of each restriction enzyme according to the manufacturer's recommendations. Three microliters of each restriction digest was mixed with 2 μl of glycerol dye loading buffer, and the mixture was electrophoresed on a 0.30-mm-thick 5% acrylamide native gel with 1× TBE. The gel was autoradiographed for 24 to 72 h on Kodak X-Omat X-ray film.

RESULTS

Ribotyping. Two completely distinct ribotypes were observed after restriction with, respectively, *Bgl*II, *Eco*RI, and *Hin*dIII. With *Bgl*II (Fig. 1A), strains of the hardjoprajitno genotype exhibited a single pattern consisting of four fragments of 9.8, 4.5, 4.1, and 2.6 kb. The single ribotype of the hardjobovis strains included three fragments of 16.4, 14.7, and 3.4 kb. With *Eco*RI (data not shown), the hardjoprajitno strains presented a single pattern of four fragments (5.6, 3.6, 1.1, and 0.8 kb), similar to the pattern of the reference strain of serovar hardjo studied previously (24). Hardjobovis strains had a single pattern of five fragments (6.2, 4.5, 2.4, 1.5, and 0.8 kb), similar to the pattern previously observed with the strain Sponselee (25). Lastly, after a *Hin*dIII restriction (Fig. 1B), hardjoprajitno strains were characterized by a single pattern of four fragments (12, 9, 2.6, and 2.3 kb), while hardjobovis strains presented a pattern of five fragments (13.6, 8.3, 4.7, 4,

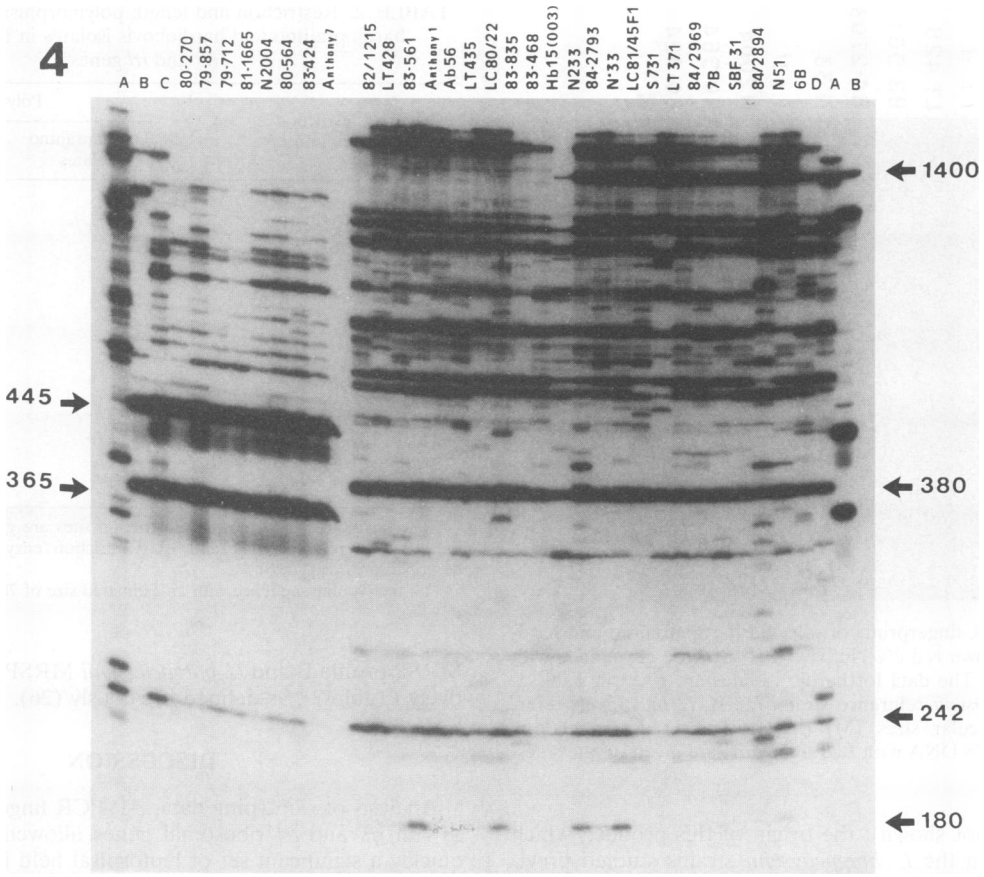


FIG. 3 and 4. Autoradiograms of AP-PCR fingerprints of genomic DNAs from selected hardjo isolates produced with primer KF (Fig. 3) and RSP (Fig. 4). The data for hardjo isolates are given in Table 1. Lanes A, *L. borgpetersenii* reference strain; lanes B, *L. interrogans* reference strain; lane C, strain Hardjoprajitno (reference strain from serovar hardjo); lane D, strain Sponselee (genotype hardjobovis). Molecular sizes (in base pairs) determined by digestion of pBR322 DNA with *Bst*NI are given on the sides.

and 2.9 kb). The banding patterns characteristic of all the ribotypes are schematically represented in a normalized graph (Fig. 2).

AP-PCR. AP-PCR fingerprints of the leptospires were obtained with the seven primers tested. The level of complexity of the patterns was variable, depending on the primer used. The patterns were simple when KpnR and SP were used and were relatively complex when KF, KG, KN, KZ, and RSP were used. In each case, strains belonging to either hardjoprajitno or hardjobovis genotypes could be separated by the presence of one to nine prominent products specific to each of the two groups. The fingerprints obtained with primers KF and RSP are presented in Fig. 3 and 4. As demonstrated previously (25a, 26), prominent species-specific products (not shared with the other leptospiral genospecies) were observed. With KF (Fig. 3), the patterns of the strains belonging to the hardjoprajitno group included the three *L. interrogans* species-specific fragments (900, 330, and 200 bp); conversely, the fingerprints of the strains related to the hardjobovis group exhibited the four *L. borgpetersenii* species-specific fragments (410, 357, 247, and 240 bp). With RSP (Fig. 4 and 5), fragments of 445 and 365 bp, which are specific for *L. interrogans* species, were present in the hardjoprajitno group fingerprints, while the fragment of 380 bp, which is specific for *L. borgpetersenii* species, was present in the hardjobovis group fingerprints.

Variations in other products were observed on sequencing gels between strains of the two groups and within each group. Additional fragments were constant within each of the two groups, giving evidence of their relative homogeneities at the subspecies level (Fig. 3 and 4). Nevertheless, the hardjobovis group could be divided into two subgroups on the basis of the presence or absence of a 1,400-bp fragment in RSP fingerprints. A detailed study of the RSP fingerprints at the strain level (Fig. 4), within the species- or group-specific fragments, demonstrated a very high level of heterogeneity inside the hardjobovis group among fragments larger than 380 bp. Conversely, the hardjoprajitno RSP fingerprints were homogeneous, with only minor variations. Similar observations were noted with KF and KG fingerprints.

Lastly, the analysis of AP-PCR produced with RSP in an agarose gel (Fig. 5) allowed a simple delineation of the two groups at the species level (with the fragments of 445 and 365 bp and of 380 bp) and of the two subgroups of the hardjobovis strains (with the 1,400-bp fragment).

MRSPs in PCR-amplified *rrs* and *rrl* genes. As demonstrated previously (26), the primers used in the present study amplified conserved portions of the *rrs* (from bases 11 to 1507) (12) and *rrl* (from bases 220 to 1466) genes (11). By PCR amplification of the *rrs* gene, one product of the expected size (1.5 kb) was found for all strains of the hardjoprajitno genotype and the reference strain of *L. interrogans*. An additional product of 260 bp was found for all strains of the hardjobovis

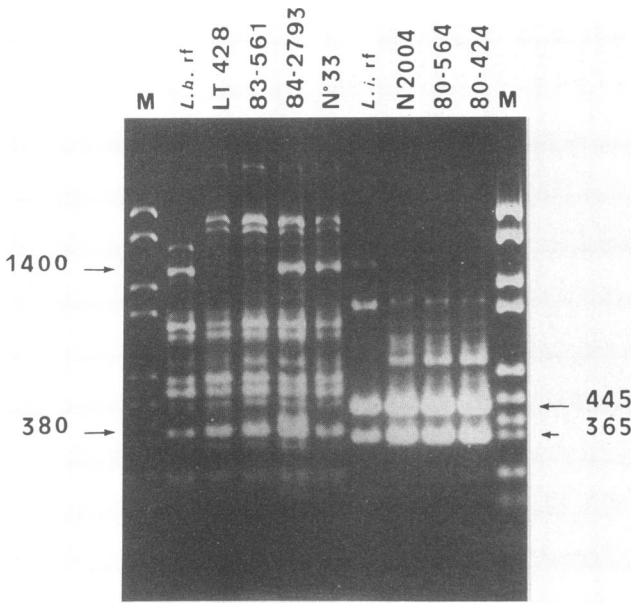


FIG. 5. AP-PCR fingerprints of selected hardjoprajitno and hardjobovis strains. Shown is a 2% NuSieve (3:1)-agarose gel stained with ethidium bromide. The data for hardjo isolates are given in Table 1. *L.b.* rf, *L. borgpetersenii* reference strain; *L.i.* rf, *L. interrogans* reference strain. Molecular sizes (M) (in base pairs) determined by digestion of pBR328 DNA with *Bgl*I and *Hin*fI are given on the sides.

genotype (data not shown); the origin of this product, which was found among the *L. borgpetersenii* strains studied previously (26), is unknown. For the *rrl* gene, an intervening sequence with an estimated size of 700 bp was present on all strains of the hardjobovis group, as demonstrated previously for MRSP profiles of *L. borgpetersenii* groups C and D (26).

For the *rrs* gene, restriction digestions performed with *Dde*I, *Hha*I, *Nla*IV, and *Taq*I allowed the hardjo isolates to be assigned to groups at the genospecies level. The results of the restriction digestions are summarized in Table 2. The hardjoprajitno strains were demonstrated to belong to *L. interrogans*, while hardjobovis isolates were characterized as *L. borgpetersenii*. The map locations of the polymorphic restriction sites in the *rrs* gene were deduced from a known *rrs* gene sequence (12). As an example, the *Nla*IV and *Hha*I digests of the PCR products from the *rrs* gene are presented in Fig. 6. One of five *Nla*IV sites was polymorphic, at base 165. When the site at base 165 is present, two fragments, of 201 (marked B in Fig. 6A) and 169 bp (marked C in Fig. 6A), can be seen, as is the case for the *L. interrogans* reference strain and the isolates from the hardjoprajitno group; the fragment of 370 bp (marked A in Fig. 6A) is due to the loss of this site and is present in the restriction pattern of the *L. borgpetersenii* reference strain and the isolates of the hardjobovis group. With *Hha*I, isolates of the hardjoprajitno group presented a restriction map of the *L. interrogans* type (two fragments of 1030 [marked A′ in Fig. 6B] and 433 bp), while isolates of the hardjobovis group were of the *L. borgpetersenii* type (the gain of a site at base 222, producing the cleavage of the 1,030-bp fragment [marked A′ in Fig. 6B] in two fragments of 852 [marked B′ in Fig. 6B] and 178 bp [marked C′ in Fig. 6B]; the restriction site at base 640 was absent). The *rrs* restriction data and restriction digestions performed with *Hha*I, *Hph*I and *Mnl*I for the *rrl* gene (data not shown) allowed the hardjoprajitno and hardjobovis isolates to be assigned to the *L. interrogans* MRSP profile B and *L. borgpetersenii* MRSP profile C, respectively (Table 2), as defined previously (26).

TABLE 2. Restriction and length polymorphisms detected among hardjoprajitno and hardjobovis isolates in PCR-amplified *rrs* and *rrl* genes[a]

Gene and restriction enzyme (position)	Polymorphism	
	Hardjoprajitno isolates	Hardjobovis isolates
rrs (16S)		
*Dde*I (80/121/216)	+/+/−	+/−/−
*Hha*I (222/640)	−/−	+/−
*Hin*fI (198/974)	−/+	−/+
*Nla*IV (165)	+	−
*Taq*I (826)	−	−
rrl (23S)		
*Hha*I (1110)	+	−
*Hph*I (626/697/788)	+/−/−	+/+/+
*Mnl*I (259/279)	+/+	+/+
*Mnl*I (377/543)	+/−	+/+
IS[b]	−	+

[a] The positions of polymorphic restriction sites are given for the sequences presented in references 11 and 12. Restriction enzymes were selected as described previously (26).

[b] IS, intervening sequence with an estimated size of 700 bp (26).

DISCUSSION

Analysis of ribotyping data, AP-PCR fingerprints, and MRSPs in *rrs* and *rrl* ribosomal genes allowed us to categorize quickly a significant set of leptospiral field isolates of serovar hardjo at the genospecies and subspecies levels. Furthermore, on the basis of the AP-PCR fingerprints, genomic heterogeneity was found among the hardjobovis strains. This approach could be used in molecular epidemiology studies. Both approaches, that is, analysis of highly conserved genes (ribotyping and MRSP) and the whole genome (AP-PCR), gave consistent results.

The genomic diversity of strains of serovar hardjo was first demonstrated by REA of DNA (29, 31), leading to the delineation of two genotypes, hardjoprajitno and hardjobovis. Differences in the pathogenicities of the two groups have been suggested (23), and the epidemiology of hardjobovis infections is variable, depending on the geographical region from which the strains are derived (42). The delineation of these two groups was confirmed by studying antigenic structures (21) and by using recombinant probes (20, 30, 33).

The extent of their diversity at the genospecies level is unknown. Since the delineation of genospecies in the genus *Leptospira*, few hardjo strains have been studied by DNA-DNA hybridization. The reference strain of serovar hardjo (strain Hardjoprajitno) was included in *L. interrogans* sensu stricto by Yasuda et al. (39), and one hardjobovis field isolate was subsequently assigned to *L. borgpetersenii* by Ramadass and Marshall (28). The present study, conducted with a significant number of serovar hardjo strains from various geographical origins, confirms the heterogeneity of this serovar at the genospecies level. MRSPs in PCR-amplified ribosomal genes and AP-PCR fingerprints gave evidence that strains belonging to the hardjoprajitno group are included in the species *L. interrogans*. On the other hand, hardjobovis strains are characterized as *L. borgpetersenii*. Ribotyping data were consistent with the MRSP findings and indicated similar species assignations on the basis of the available database (24, 25). These

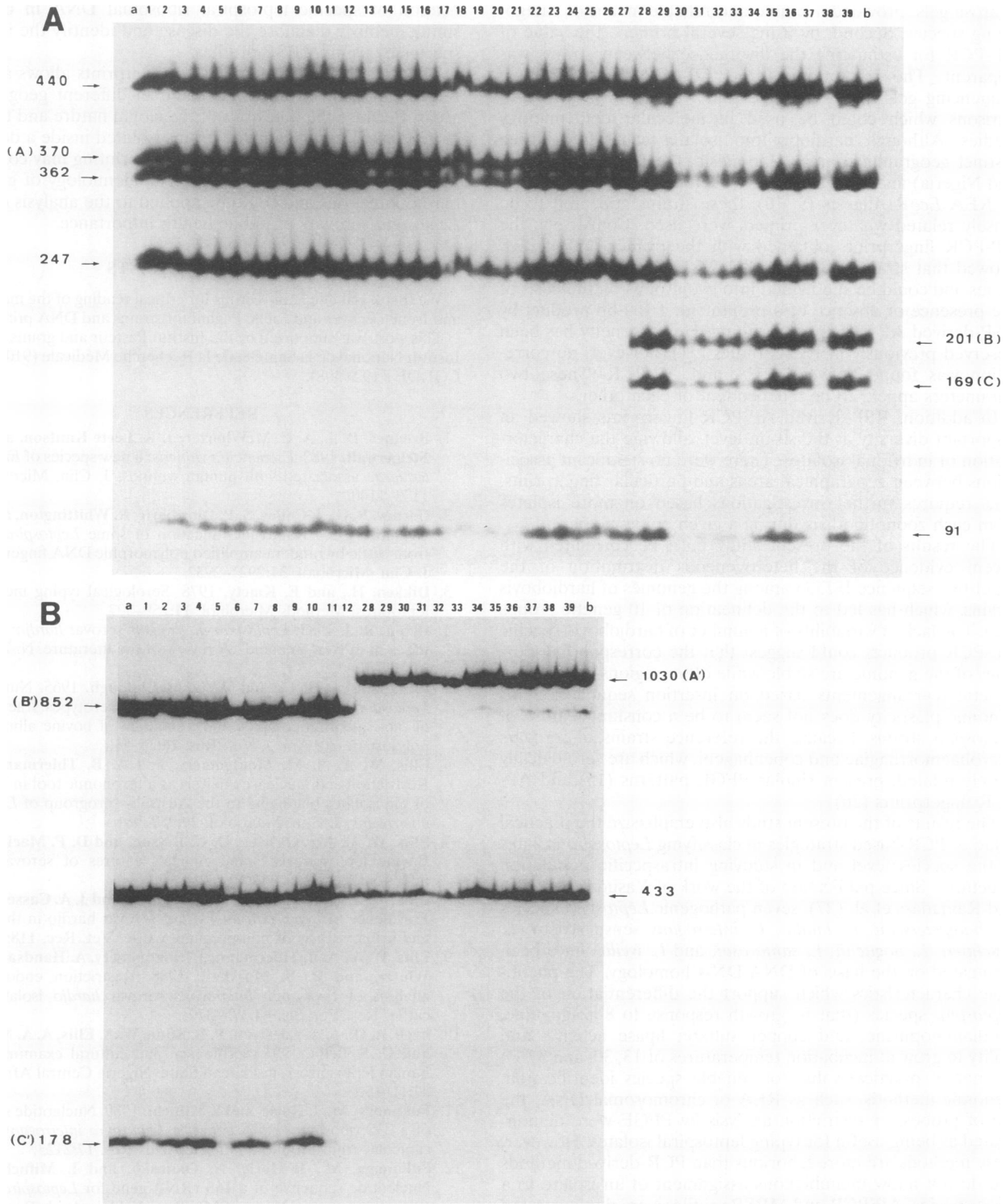


FIG. 6. Autoradiograms of restriction digests with *Nla*IV (A) and *Hha*I (B) of PCR-amplified 16S genes. The lane assignments for hardjo field isolates are given in Table 1. Lanes a, *L. interrogans* reference strain; lanes b, *L. borgpetersenii* reference strain. Molecular sizes (in base pairs) determined by digestion of pBR322 DNA with *Bst*NI digest are given on the sides. Restriction fragments labeled A, B, C, and A′, B′, C′ are discussed in the text.

findings are corroborated by those of Corney et al. (2), who have independently characterized hardjobovis isolates as *L. borgpetersenii* by using random amplification of polymorphic DNA (38).

The dual value of using AP-PCR fingerprints for the characterization of leptospiral isolates is illustrated in the present study. First, at the species level, the presence of specific prominent PCR products, which could be easily observed in

agarose gels, provided a simple and reliable means of categorizing species. Second, by using several primers, the value of AP-PCR for estimating the divergence between strains was apparent. The resolution of the AP-PCR fingerprints on sequencing gels makes possible significant intraspecies comparisons which could be used in molecular epidemiology studies. Although hardjoprajitno isolates came from three distinct geographical areas (Indonesia, the United Kingdom, and Nigeria) and differences have been described on the basis of REA *Eco*RI digests (9, 10), these strains appeared to be closely related whatever primers were used. Conversely, the AP-PCR fingerprints obtained with the primer RSP or KF showed that strains of the hardjobovis group were heterogeneous and could be subdivided into two groups on the basis of the presence or absence of a prominent, 1,400-bp product by RSP-derived AP-PCR fingerprinting. Heterogeneity has been observed previously in REA studies (31); however, no correlation was found between REA and AP-PCR. These two parameters appear to be independent of each other.

In addition, RSP-derived AP-PCR fingerprints showed an important diversity at the strain level, allowing the characterization of individual isolates. There were no significant associations between geographical areas and particular fingerprints. This requires further investigations based on more isolates from each zoonotic focus during a given epidemic period.

The results of the present study must be correlated with recent evidence of the heterogeneous distribution of the repetitive sequence IS*1533* among the genomes of hardjobovis strains, which has led to the delineation of 10 genetic groups (42). The lack of variability of a number of hardjobovis-specific AP-PCR products could suggest that the corresponding portions of the genome are stable while other regions can present genetic rearrangements based on insertion sequences. This genomic plasticity does not seem to be a constant feature of *Leptospira* strains, because the reference strains of serovars icterohaemorrhagiae and copenhageni, which are serologically closely related, present similar PFGE patterns (15) and AP-PCR fingerprints (26).

The results of the present study also emphasize the practical value of PCR-based strategies in classifying *Leptospira* isolates at the species level and in studying intraspecific population structures. Since publication of the work of Yasuda et al. (39) and Ramadass et al. (27), seven pathogenic *Leptospira* species (*L. borgpetersenii*, *L. inadai*, *L. interrogans* sensu stricto, *L. kirschneri*, *L. noguchii*, *L. santarosai*, and *L. weilii*) have been delineated on the basis of DNA-DNA homology. The phenotypic characteristics which support the differentiation of the *Leptospira* species (that is, growth response to 8-azaguanine, 2,6-diaminopurine, and copper sulfate; lipase activity; and ability to grow at incubation temperatures of 13, 30, and 37°C) are not of practical value for reliable species identification. Genomic methods, such as REA of chromosomal DNA, the use of probes, or restriction analysis by PFGE were demonstrated as being useful for typing leptospiral isolates. However, these methods are more laborious than PCR-derived methods and do not allow unambiguous assignment of an isolate to a given species. AP-PCR and MRSP profiles were demonstrated to be effective tools for the rapid determination of the genospecies of *Leptospira* isolates, which is the first step in bacterial identification. Furthermore, AP-PCR or MRSPs need much less DNA in comparison with the amount needed for DNA-DNA hybridization. This is an important consideration for slowly growing bacteria such as leptospires. Finally, determination of MRSPs in *rrs* and *rrl* ribosomal genes should be applicable without strain isolation, which is not always possible for *Leptospira* isolates. Work is in progress to allow an amplification of specific leptospiral ribosomal DNA in order to simultaneously diagnose the disease and identify the infecting species by using MRSP profiles.

The detailed study of AP-PCR fingerprints allows an accurate comparison of strains isolated in different geographical areas. Because the questions of the clonal nature and the level of genomic heterogeneity of strains isolated inside a delimited enzootic focus remain, AP-PCR fingerprinting may contribute to the knowledge of the molecular epidemiology of genotype hardjo infections and could be applied to the analysis of other *Leptospira* serovars of public health importance.

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