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Rapid Identification of Pathogenic *Leptospira* Species (*Leptospira interrogans*, *L. borgpetersenii*, and *L. kirschneri*) with Species-Specific DNA Probes Produced by Arbitrarily Primed PCR

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Arbitrarily primed PCR (AP-PCR) assays can be used to discriminate between species of *Leptospira*. Comparative analysis of the fingerprints obtained from representative sets of serovar reference strains of *Leptospira interrogans* sensu stricto, *L. borgpetersenii*, and *L. kirschneri* and the reference strains of the other *Leptospira* spp. revealed species-specific DNA fragments. These species-specific sequences were reamplified in order to produce digoxigenin-11-dUTP-labeled genomic DNA probes that could be used to identify *Leptospira* species. Three probes (specific for *L. interrogans* sensu stricto, *L. borgpetersenii*, and *L. kirschneri*) were selected and tested with 72 representative serovar reference strains, all of which had previously been studied by DNA-DNA hybridization. The two techniques were in general agreement, and hybridization with AP-PCR-derived probes was shown to be a useful approach for rapid species determination of leptospires, without the prior need for DNA sequence information. These nonradioactive probes can be used to identify *Leptospira* species in nonspecialized laboratories, and this should contribute to a better knowledge of the molecular epidemiology of leptospirosis.

Leptospirosis, which is caused by pathogenic members of the genus *Leptospira*, is a zooanthroponosis widespread throughout the world. Pathogenic leptospires were originally classified on the basis of their antigenic characteristics. A single nomenspecies, *Leptospira interrogans* sensu lato, was divided into taxa called serovars by a classification based on pairwise comparison of the extent of cross-absorption of rabbit antisera against each serovar (5). Antigenically related serovars constitute serogroups; in total, *L. interrogans* sensu lato included at least 202 serovars in 23 serogroups (12). However, the identification of isolates into serovars is tedious, requiring the maintenance of a comprehensive collection of strains and the corresponding rabbit immune sera (11).

Molecular taxonomic studies have profoundly improved our knowledge of the diversity of *Leptospira* spp. DNA-DNA hybridization studies (21, 28) showed that members of *L. interrogans* sensu lato were very diverse at the DNA level. This led to pathogenic leptospires being grouped into six (21) and then eight (28) species: *L. borgpetersenii*, *L. inadai*, *L. interrogans* sensu stricto, *L. kirschneri*, *L. meyeri*, *L. noguchii*, *L. santarosai*, and *L. weilii.*

Several molecular tools can be used as additional typing systems in epidemiological studies, since they identify strain differences at the subspecies level. These include restriction endonuclease analysis of chromosomal DNA by fixed-field gel electrophoresis (7, 24) or pulsed-field gel electrophoresis (10), DNA hybridization using repetitive sequences (16), and ribotyping (17, 18).

Nevertheless, the identification of leptospires at the species level remains difficult, as DNA-DNA hybridization cannot be used for routine identification. PCR-based strategies were developed to categorize the new species of *Leptospira* (20). Mapped restriction site polymorphisms (MRSPs) in PCR-amplified ribosomal genes allowed reference strains to be grouped in their species, in agreement with DNA relatedness studies. Arbitrarily primed PCR (AP-PCR) generates distinctive fingerprints that can be used for molecular epidemiology and for rapid identification of isolates at the species level through the presence of prominent species-specific PCR products. However, these methods require the use of radiolabeled nucleotides, and their use is restricted to reference laboratories. This makes it very difficult to perform epidemiological studies to determine the ecological significance of these new species, including geographical distributions and the clinical aspects of related diseases. There is a need for simple and reliable tools to allow rapid identification of leptospiral field isolates at the species level.

The purpose of this study was to develop a strategy for producing nonradioactive DNA probes able to discriminate among the pathogenic *Leptospira* species. DNA-DNA hybridization data demonstrated the high level of divergence between *Leptospira* species (28), and this was also demonstrated by using AP-PCR fingerprinting (20). Each species was characterized by prominent AP-PCR products that were not shared with the other species and which were identified as potential genetic markers at the species level (19, 20). In the present report, we describe the production of species-specific probes derived from these highly conserved amplicons to identify the three pathogenic species—*L. interrogans* sensu stricto, *L. borgpetersenii*, and *L. kirschneri*—which make up two-thirds of the *Leptospira* spp. documented by DNA-DNA hybridization (3, 21, 28). In addition, these three species contain many serovars of worldwide public health importance, for example, *L. inter-*

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rogans sensu stricto serovars australis, bratislava, icterohaemorrhagiae, pomona, and pyrogenes, *L. borgpetersenii* serovars ballum, javanica, hardjo type Hardjobovis, and sejroe, and *L. kirschneri* serovars bim, cynopteri, and grippotyphosa (12). The probes were selected by using a limited set of reference strains, and then their specificity was confirmed by using 70 serovar reference strains, previously documented at the species level by DNA-DNA hybridization, and also 14 field isolates.

MATERIALS AND METHODS

Bacterial strains. (i) Reference strains. A total of 72 reference strains of the family *Leptospiraceae*, from the Collection of the WHO Collaborating Center for Leptospirosis, Institut Pasteur, Paris, France, were included in this study and are listed in Table 1. Sixty-nine were pathogenic *Leptospira* reference strains; among them were the type strains of the eight species previously included in *L. interrogans* sensu lato: *L. borgpetersenii*, *L. inadai*, *L. interrogans* sensu stricto, *L. kirschneri*, *L. meyeri*, *L. noguchii*, *L. santarosai*, and *L. weilii*. In addition, representative serovar reference strains were selected according to their epidemiological importance: 14 *L. borgpetersenii* strains, 16 *L. interrogans* strains, 20 *L. kirschneri* strains, 5 *L. noguchii* strains, 3 *L. santarosai* strains, and 3 *L. weilii* strains. This study included the type strain of the saprophytic species *L. biflexa* and type strains of the related genera *Leptonema* (*Leptonema illini*) and *Turneria* (*Turneria parva*), which were tested as closely related nonleptospiral spirochetes.

(ii) Wild-type isolates. Fourteen field isolates, assigned to their respective species by using AP-PCR and MRSP, were hybridized with the selected probes. Within *L. borgpetersenii* were strains of serovar hardjo type Hardjobovis (83-561, 83-468, 84-2793, and Kidney 34) (19) and one ballum isolate (Ro95) from New Caledonia. Within *L. interrogans* were serovar icterohaemorrhagiae (At95), pomona (Ma95 and Se95), and pyrogenes (Di95) strains isolated in New Caledonia. Within *L. kirschneri* were serovar grippotyphosa strains AM2, VM23, Duyster, Kuthy, and Turna.

Culture conditions and preparation of DNA. Strains were grown in EMJH medium (6) at 30°C with shaking until the stationary phase of growth was reached. Two different DNA purification methods were used. (i) For all the reference strains, chromosomal DNA was extracted by using a phenol-chloroform method as described by Brenner et al. (4). (ii) To test a more practical method, DNA from the species reference strains and wild-type strains was also purified by using silica particles and guanidium thiocyanate lysis buffer according to the 2-h method described by Boom et al. (1).

AP-PCR. AP-PCR was performed with the purified total genomic DNA as described elsewhere (19, 26) by using 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′). In addition, considering the G+C content of *Leptospira* spp. (28), two AT-rich primers were tested: PR1 (5′-CGC TAA AAC TAA TAT CAT GA-3′) and PR2 (5′-GGA AAC TTA CAC TAA CAG-3′). The primers 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 deoxynucleotide triphosphate, 1 μM single nucleotide primer, 5 μCi of 3,000-Ci/mmol [^{32}P]dCTP (Amersham, Little Chalfont, Buckinghamshire, England), and 1.25 U of *Taq* polymerase (Amersham). The reaction mixtures were cycled in a GeneAmp 9600 PCR machine (Perkin-Elmer), twice 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 2 min for extension). Five microliters of each reaction mixture was combined with 15 μl of 98% formamide-dye and heated to 68°C for 15 min; 3 ml of each sample was loaded on 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 *Bst*NI digests (New England Biolabs) and pBR322 *Msp*I digests (New England Biolabs, Beverly, Mass.) were used as molecular size markers. The gel was autoradiographed for 24 to 48 h on Kodak X-Omat X-ray film. Hot-ink marks allowed the locating of the species-specific amplicons.

Probe isolation and labeling. Species-specific amplicons were selected from the autoradiograms of the AP-PCR patterns as follows. For the first screening, a set of 15 reference strains was used, including six serovars from the species for which the specific probe was searched, the type strains of the seven other pathogenic species, and the saprophytic strains Patoc I (*L. biflexa* type strain) and 3055 (*Leptonema illini* type strain). The presumed species-specific amplicons were excised from the polyacrylamide gel and eluted in TE buffer as described by Sambrook et al. (22). The amount and quality of purified DNA were evaluated by using a 1.5% agarose electrophoresis gel (NuSieve 3:1; FMC Corp., Rockland, Maine).

Probes were produced with a specific and stringent PCR program (8), using the same primer that gave the selected matrix, and simultaneously labeled by incorporation of digoxigenin-11-dUTP (Boehringer, Mannheim, Germany). Fifty-microliter reaction mixtures were prepared with 2 to 5 μl of purified DNA (according to the evaluated amount of DNA in the solution)–1× *Taq* polymerase buffer (100 mM Tris [pH 8.3], 500 mM KCl)–1.5 mM $MgCl_2$–0.2 mM each dATP, dCTP, and dGTP–0.18 mM dTTP–20 μM digoxigenin-11-dUTP–0.5 μM single nucleotide primer–1.25 U of *Taq* polymerase (Amersham). The PCR was performed in a GeneAmp 9600 (Perkin-Elmer) PCR machine as follows: 1 cycle with denaturation at 94°C for 2 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min 30 s; 28 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min 30 s; and 1 cycle with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 10 min. The homogeneity of the products was evaluated on a 1.5% agarose gel (NuSieve 3:1; FMC Corp.).

Dot blot hybridization with digoxigenin-11-dUTP-labeled probes. For each strain tested, 300 ng of purified leptospiral DNA was denatured by heating at 96°C for 10 min and spotted onto Hybond-N nylon membranes (Amersham) using a Hybri-Slot Manifold apparatus (Gibco BRL). Homologous DNA from the corresponding species reference strain was used as a positive control, and distilled water was used as a negative control. DNA was then fixed onto the filter by UV treatment of 120 mJ/cm^2 for 3 min on a Spectro-Linker XL-1500 UV Crosslinker (Spectronics Corporation, New York, N.Y.).

The specificity of the selected probe was first tested on the set used for its selection (15 reference strains) and, where there was a perfect match, on all 57 remaining strains included in the study. The hybridization experiments were done in triplicate. Prehybridization and hybridization temperatures were 61°C for detection of *L. interrogans* strains, 65°C for *L. kirschneri*, and 63°C for *L. borgpetersenii*. All filters were prehybridized for 1 h in 5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate)–30% formamide–0.5% blocking reagent (Boehringer)–0.1% *N*-lauroylsarcosine–0.02% sodium dodecyl sulfate. Hybridization was carried out overnight with 5 ml of heat-denatured probe in the same buffer per 100 cm^2 of membrane. Detection was achieved by using an antidigoxigenin antibody conjugated to alkaline phosphatase and disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD) as a substrate (Boehringer) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

In previous studies, the value of AP-PCR fingerprinting for identifying *Leptospira* isolates at the species level (20) and for molecular epidemiology of serovars of public health importance (19) was demonstrated. For a given primer, each species gave prominent AP-PCR fingerprinting products not shared with the other species (Fig. 1). This allowed us to design and construct species-specific nonradioactive probes for rapid identification of *Leptospira* isolates.

Identification of species *L. interrogans*. Initially, four AP-PCR products showed potential for the identification of *L. interrogans*. After reamplification and nonradioactive labeling, one amplicon derived from primer RSP (of approximately 390 bp and designated probe $RSPI_2$) gave, under stringent conditions, a specific matching with the DNAs of all 17 *L. interrogans* strains which were tested. Cross-hybridization with the saprophytic species *L. biflexa* or the related spirochetes (*Leptonema illini* and *T. parva*) or with the 52 remaining pathogenic *Leptospira* strains was not observed. An exception was the *L. meyeri* reference strain (serovar ranarum strain ICF), which produced a positive hybridization signal. The probe was satisfactory when tested on filters spotted with DNAs of *L. interrogans* strains extracted by both methods described above. Results obtained with the DNAs of 18 strains isolated by the silica method (1) are illustrated in Fig. 2.

Discrepancies are reported in the literature about the species assignments of some serovars (21, 28). In this study, cross-hybridization of the $RSPI_2$ probe with serovar ranarum strain ICF was observed. Yasuda et al. (28) assigned this serovar to *L. meyeri*, defined as a species distinct from *L. interrogans*, whereas previous studies based on DNA-DNA hybridization data (2), phenotypic characteristics (23), and AP-PCR fingerprints and MRSP profiles (20) did not. Similarly, serovar muenchen is here assigned to *L. interrogans*, and this result is consistent with ribotyping (18), as there is a strict identity

TABLE 1. *Leptospira* strains used in this study

Genospecies	Serovar	Strain	Serogroup[f]	Source	Hybridization with probe:		
					$RSPI_2$	$PR1B_3$	KGK_1
L. biflexa	patoc[a,c]	Patoc I	Sem.	Water	−	−	−
L. borgpetersenii	balcanica[b,c,d]	1627 Burgas	Sej.	Human	−	+	−
	ballum[a,c]	Mus 127	Bal.	Field mouse	−	+	−
	castellonis[c,d]	Castellòn 3	Bal.	Wood mouse	−	+	−
	dehong[d]	DE 10	Jav.	*Suncus murinus*	−	+	−
	hardjo[b,c,d]	Hardjobovis Sponselee	Sej.	Cow	−	+	−
	istrica[c,d]	Bratislava	Sej.	Wood mouse	−	+	−
	javanica[a,b,c]	Veldrat Batavia 46	Jav.	Field rat	−	+	−
	jules[c,d]	Jules	Heb.	Human	−	+	−
	kenya[c,d]	Njenga	Bal.	Pouched rat	−	+	−
	mini[a,b,c]	Sari	Min.	Human	−	+	−
	nona[c,d]	Nona	Heb.	Human	−	+	−
	sejroe[a,c]	M 84	Sej.	Mouse	−	+	−
	sofia[c,d,e]	Sofia 874	Jav.	Human	−	+	−
	sorexjalna[c,d]	Sorex Jalná	Jav.	Shrew	−	+	−
	tarassovi[a,b,c]	Perepelitsin	Tar.	Human	−	+	−
L. inadai	lyme[a]	10	Lym.	Human	−	−	−
L. interrogans	australis[a,b,c]	Ballico	Aus.	Human	+	−	−
	bangkinang[c,d]	Bankinang I	Aut.	Human	+	−	−
	bratislava[b,c,d]	Jez-Bratislava	Aus.	Hedgehog	+	−	−
	carlos[c,d]	C 3	Aut.	Toad	+	−	−
	djasiman[a,b,c]	Djasiman	Dja.	Human	+	−	−
	fugis[b,c,d]	Fudge	Aus.	Human	+	−	−
	geyaweera[c,d]	Geyaweera	Sej.	Human	+	−	−
	hawain[c,d]	LT 62-68	Aus.	Bandicoot	+	−	−
	hebdomadis[a,b,c]	Hebdomadis	Heb.	Human	+	−	−
	icterohaemorrhagiae[a,b,c]	RGA	Ict.	Human	+	−	−
	jalna[a,b,c]	Jalná	Aus.	Yellowthroat	+	−	−
	malaya[c,d,e]	H6	Can.	Human	+	−	−
	muenchen[b,c,d]	München C 90	Aus.	Human	+	−	−
	pomona[a,b,c]	Pomona	Pom.	Human	+	−	−
	pyrogenes[a,b,c]	Salinem	Pyr.	Human	+	−	−
	rachmati[c,d]	Rachmat	Aut.	Human	+	−	−
	sentot[c,d]	Sentot	Dja.	Human	+	−	−
L. kirschneri	agogo[d]	Agogo	Dja.	Human	−	−	+
	bim[c,d]	1051	Aut.	Dog	−	−	+
	bafani[c,d]	Bafani	Can.	Human	−	−	+
	bogvere[c,d]	LT 60-69	Ict.	Rat	−	−	+
	butembo[b,d]	Butembo	Aut.	Human	−	−	+
	cynopteri[a,b,c]	3522 C	Cyn.	Bat	−	−	+
	djatzi[c,d]	HS 26	Bat.	Human	−	−	+
	erinaceiauriti[c,d]	Erinaceus auritus 670	Aut.	Hedgehog	−	−	+
	galtoni[c,d]	LT 1014	Can.	Cow	−	−	+
	grippotyphosa[b,c,d]	Moskva V	Gri.	Human	−	−	+
	kambale[b,c,d]	Kambale	Heb.	Human	−	−	+
	kamituga[c,d]	Kamituga	Can.	Human	−	−	+
	kunming[d]	K 5	Pom.	*Apodemus chevrieri*	−	−	+
	lambwe[c,d]	Lambwe	Aut.	Unstripped grass rat	−	−	+
	mozdok[c,d]	5621	Pom.	Field vole	−	−	+
	mwogolo[c,d]	Mwogolo	Ict.	Human	−	−	+
	ndahambukuje[c,d]	Ndahambukuje	Ict.	Human	−	−	+
	ndambari	Ndambari	Ict.	Human			
	ramisi[b,c,d]	Musa	Aus.	Human	−	−	+
	ratnapura[c,d]	Wumalasena	Gri.	Human	−	−	+
	vanderhoedeni[c,d]	Kipod 179	Gri.	Hedgehog	−	−	+
L. meyeri	ranarum[a]	ICF	Ran.	Frog	+	−	−
L. noguchii	bajan[d]	Toad 60	Aus.	Toad	−	−	−
	carimagua[d]	9160	She.	Unknown	−	−	−
	claytoni[d]	1348 U	Bat.	Spiny rat	−	−	−
	louisiana[a,b,c]	LSU 1945	Lou.	Armadillo	−	−	−
	nicaragua[d]	1011	Aus.	*Mustela nivalis*	−	−	−
	panama[a,c]	CZ 214	Pan.	Opossum	−	−	−

Continued on following page

TABLE 1—*Continued*

Genospecies	Serovar	Strain	Serogroup[f]	Source	Hybridization with probe:		
					$RSPI_2$	$PR1B_3$	KGK_1
L. santarosai	alexi[c,d]	HS 616	Pyr.	Human	–	–	–
	canalzonae[c,d]	CZ 188	Gri.	Spiny rat	–	–	–
	fluminense[d]	Aa 3	Jav.	Field mouse	–	–	–
	shermani[a,b,c]	1342 K	She.	Spiny rat	–	–	–
L. weilii	anhoa[c,d,e]	LT 90-68	Cel.	Human	–	–	–
	celledoni[a,b,c]	Celledoni	Cel.	Human	–	–	–
	coxi[d]	Cox	Jav.	Human	–	–	–
	whitcombi[c,d,e]	Whitcomb	Cel.	Human	–	–	–
Leptonema illini	illini	3055	Lep.	Cow	–	–	–
T. parva	parva	H	Tur.	Media	–	–	–

[a] Assignment of species was done as described by Yasuda et al. (28).
[b] Assignment of species was done as described by Ramadass et al. (21).
[c] Assignment of species was done as described by Perolat et al. (18).
[d] Assignment of species was done as described by Brenner et al. (3).
[e] Conflicting species assignments (serovars, malaya, muenchen, anhoa, and whitcombi) are discussed in the text.
[f] Aus., Australis; Aut., Autumnalis; Bal., Ballum; Bat., Bataviae; Can., Canicola; Cel., Celledoni; Cyn., Cynopteri; Dja., Djasiman; Gri., Grippotyphosa; Heb., Hebdomadis; Ict., Icterohaemorrhagiae; Jav., Javanica; Lou., Louisiana; Lym., Lyme; Min., Mini; Pan., panama; Pom., Pomona; Pyr., Pyrogenes; Ran., Ranarum; Sej., Sejroe; She., Shermani; Tar., Tarassovi; Sem., Semaranga; Lep., Leptonema; Tur., Turneria.

between the ribotypes of strain München C90 and of strain RGA (the type strain of *L. interrogans*). It is also consistent with the results of DNA-DNA hybridization (2, 3) but not consistent with the results of Ramadass et al. (21), who assigned this strain to *L. noguchii*. Lastly, serovar malaya was identified as belonging to *L. interrogans*, in agreement with ribotyping (18), but this was not consistent with DNA-DNA hybridization data (3), which assigned this strain to *L. inadai.*

Identification of species *L. borgpetersenii*. The fragment used as a probe for *L. borgpetersenii* was obtained from AP-PCR with primer PR1. It was approximately 680 bp, and the probe was called $PR1B_3$. Under stringent conditions, a specific matching with the DNAs of all 15 *L. borgpetersenii* strains was obtained. Cross-hybridization with the saprophytic species *L. biflexa* or the related spirochetes (*Leptonema illini* and *T. parva*) or with the 54 remaining pathogenic *Leptospira* strains was not observed (data not shown). The hybridization method was satisfactory whichever DNA purification method was used. The assignment of serovar sofia to *L. borgpetersenii* was in agreement with ribotyping (18) but not with DNA-DNA hybridization data (3). Lastly, serovars anhoa and whitcombi, which were previously characterized as *L. weilii* by their complex ribotypes (18) very close to the pattern of serovar celledoni strain Celledoni (reference strain of *L. weilii*) (17), were not recognized by probe $PR1B_3$ but were recently reported as belonging to *L. borgpetersenii* (3).

Identification of species *L. kirschneri*. The fragment used as a probe for *L. kirschneri* was obtained by AP-PCR with the KG primer. Its molecular size was approximately 760 bp, and the probe was called KGK_1. Figure 1 shows the result obtained with the AP-PCR sequencing gel from which was eluted the *L. kirschneri* species-specific fragment used for probe production. Under stringent conditions, a specific matching with the DNAs of all 21 *L. kirschneri* strains was obtained (Fig. 3). Cross-hybridization with the saprophytic species *L. biflexa* or the related spirochetes (*Leptonema illini* and *T. parva*) or with the 48 remaining pathogenic *Leptospira* strains was not observed. As with the probes specific for *L. borgpetersenii* and *L. interrogans*, the hybridization method was satisfactory regardless of the DNA purification method used.

Wild-type isolates. The 14 field isolates tested were assigned by the three probes into their respective species, as previously defined by using AP-PCR and MRSP (see Materials and Methods; data not shown).

The choice of candidate probes is the critical step of this method. AP-PCR has good reproducibility (26), as it links one short step of low-stringency amplification (2 cycles) to a longer secondary step of stringent PCR (40 cycles). The use of primers 18 or more bases long generally produces more polymorphic PCR products that can be used for characterization of species of *Leptospira* (20). Previous studies have used randomly amplified polymorphic DNA (27) with short primers (10 bases) for the selection of species-specific probes (9, 14, 15) in which DNA fragments were excised from agarose gels. To analyze AP-PCR products in a very discriminative way, we visualized fingerprints on polyacrylamide gels. This ensured that the band selected to construct the probe corresponded to a single DNA fragment and not to a doublet, as had happened in preliminary experiments with agarose gels. The criteria for choosing the fragments of interest were (i) specificity for a particular species and (ii) a strong signal on the autoradiogram. The purified DNA was then used as a matrix to construct probes in a stringent specific PCR, with the incorporation of digoxigenin-11-dUTP for further immunodetection at the hybridization step.

Several candidate probes were tested for their species specificity and for their stability at −20°C. Hybridization experiments were first performed with a set of 15 strains, including six serovars from the species for which the specific probe was selected, the type strains of the seven other pathogenic species, and the saprophytic strains Patoc I (*L. biflexa* type strain) and 3055 (*Leptonema illini* type strain). Stringent hybridization conditions were chosen to give a clear positive signal for the strains belonging to the species concerned and also a satisfactory specificity. For each species considered, one probe was selected and tested on the 72 strains included in this study: $RSPI_2$ for *L. interrogans*, $PR1B_3$ for *L. borgpetersenii*, and KGK_1 for *L. kirschneri.*

Recent studies of the taxonomy of *Leptospira* (3, 17, 18, 20, 21, 28) indicate that six reference strains included in this study

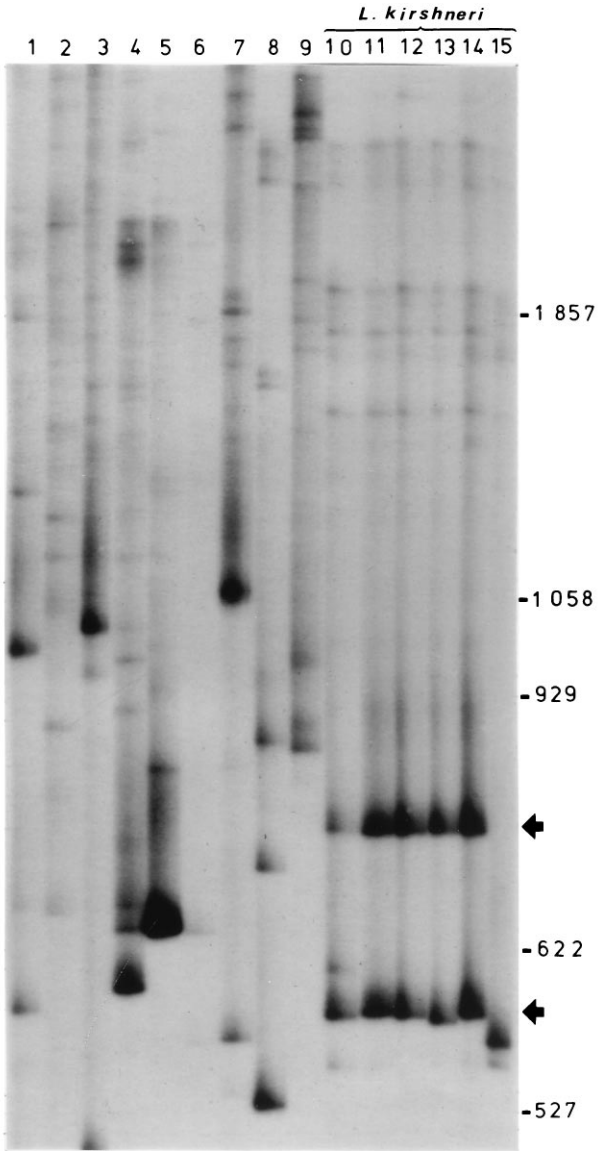

FIG. 1. AP-PCR profiles obtained with KG primer on a 4% polyacrylamide gel with 15 *Leptospira* strains, comprising six *L. kirschneri* serovars. Lane 1, serovar icterohaemorrhagiae strain RGA; lane 2, serovar javanica strain Veldrat Batavia 46; lane 3, serovar panama strain Panama; lane 4, serovar shermani strain 1342 K; lane 5, serovar celledoni strain Celledoni; lane 6, serovar ranarum strain ICF; lane 7, serovar lyme strain 10; lane 8, serovar patoc strain Patoc I; lane 9, *Leptonema illini*; lane 10, serovar cynopteri strain 3522 C; lane 11, serovar grippotyphosa strain Moskva V; lane 12, serovar mozdok strain 5621; lane 13, serovar ndambari strain Ndambari; lane 14, serovar vanderhoedeni strain Kipod 179; lane 15, serovar ramisi strain Musa. Molecular sizes (in base pairs) are indicated on the right of the gel. The species-specific bands that were excised from the gel and used as probe candidates are indicated (arrows). The upper one, of approximately 760 bp, was selected to produce the KGK_1 probe. The gel was then autoradiographed for 2 more days to verify that the bands had been correctly excised.

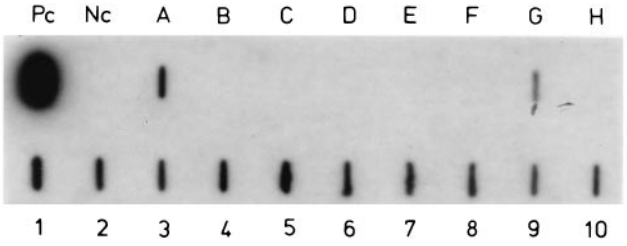

FIG. 2. Hybridization of the $RSPI_2$ probe with DNAs purified by the silica method (1). Pc, positive control (nonlabeled DNA homologous to the probe); Nc, negative control (distilled water); A, serovar icterohaemorrhagiae strain RGA; B, serovar javanica strain Veldrat Batavia 46; C, serovar cynopteri strain 3522 C; D, serovar panama strain Panama; E, serovar shermani strain 1342 K; F, serovar celledoni strain Celledoni; G, serovar ranarum strain ICF; H, serovar lyme strain 10; 1, serovar pomona strain Pomona; 2, serovar djasiman strain Djasiman; 3, serovar hebdomadis strain Hebdomadis; 4, serovar bratislava strain Jez Bratislava; 5, serovar jalna strain Jalná; 6, serovar pyrogenes strain Salinem; 7, serovar australis strain Ballico; 8, serovar hawain strain LT 62-68; 9, serovar muenchen strain München C 90; 10, serovar fugis strain Fudge.

have a controversial species assignment. Serovar ranarum strain ICF was grouped with *L. interrogans* sensu stricto strains in early DNA-DNA hybridization studies (2) but was recently considered as a new species, *L. meyeri*, with a low level of homology with other *Leptospira* spp. (28). AP-PCR and MRSP data grouped both serovars ranarum and evansi (the second serovar of serogroup Ranarum) with *L. interrogans* (20). The respective ribotypes of these two strains were closely related (18), and recently DNA-DNA hybridization assigned serovar evansi to *L. interrogans* (3). Lastly, multilocus enzyme electrophoresis confirmed this proximity of serovar ranarum strain ICF with the *L. interrogans* cluster (13). According to these discrepancies among taxonomic data, the status of the serovar ranarum strain ICF would need to be clarified.

For five other serovars, hybridization profiles with our three probes failed to correlate exactly with DNA-DNA hybridization data. Serovar sofia strain Sofia 874, attributed to *L. meyeri* (3), did not hybridize with the $RSPI_2$ probe but hybridized with $PR1B_3$. Ribotyping classified this strain in the species *L. borgpetersenii* (18), and its ribotype was identical to that of serovar nona strain Nona (18), identified as an *L. borgpetersenii* strain by DNA-DNA hybridization (3). Serovar malaya strain H6 hybridized with probe $RSPI_2$ but was provisionally classified as *L. inadai* (3). However, its ribotype was identical to these of serovars jonsis (strain Jones) and sumneri (strain Sumner) (17), which were both assigned to *L. interrogans* by DNA-DNA hybridization (3), and it was identified positively with $RSPI_2$. Lastly, serovars whitcombi (strain Whitcomb) and anhoa (strain LT 90-68), previously considered on the one hand as *L. weilii* strains according to the similarity of their ribotypes (18) and multilocus enzyme electrophoresis patterns (13) with the type strain Celledoni (17) and on the other hand as *L. borgpetersenii*

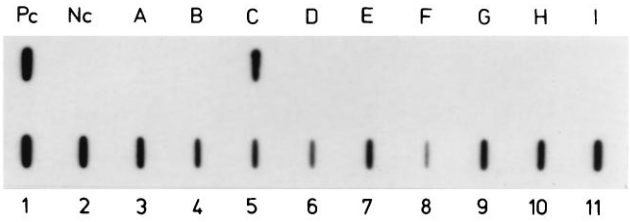

FIG. 3. Hybridization of the KGK_1 probe with strains extracted by the phenol-chloroform method (3). Pc, positive control (nonlabeled DNA homologous to the probe); Nc, negative control (distilled water); A, serovar icterohaemorrhagiae strain RCA; B, serovar javanica strain Veldrat Batavia 46; C, serovar cynopteri strain 3522 C; D, serovar panama strain Panama; E, serovar shermani strain 1342 K; F, serovar celledoni strain Celledoni; G, serovar ranarum strain ICF; H, serovar lyme strain 10; I, serovar patoc strain Patoc I; 1, serovar mozdok strain 5621; 2, serovar ndambari strain Ndambari; 3, serovar vanderhoedeni strain Kipod 179; 4, serovar ramisi strain Musa; 5, serovar mwogolo strain Mwogolo; 6, serovar bogvere strain LT 60-69; 7, serovar galtoni strain LT 1014; 8, serovar kamituga strain Kamituga; 9, serovar kambale strain Kambale; 10, serovar ratnapura strain Wumalasena; 11, serovar grippotyphosa strain Moskva V.

strains as determined by DNA-DNA hybridization (3), were not recognized by the $PR1B_3$ probe.

The uncertainties about the taxonomic positions of these six reference strains confirm previous discrepancies between the results of three major DNA-DNA hybridization studies designed to delimit species within the genus *Leptospira* (3, 21, 28). However, these cases are uncommon. For almost all the serovars previously studied, there is a general agreement between DNA-DNA hybridization data, ribotyping (17, 18), and the use of other species-specific probes (25). These cases illustrate the difficulties in efficient checking of the reference collections with the traditional serological tests and demonstrate the value of the simultaneous use of several molecular tools, such as pulsed-field gel electrophoresis (10), PCR-derived methods (19, 30), or probes (25, 29), for this purpose. A cross-comparison of the controversial serovars among the reference collections maintained in the different *Leptospira* reference laboratories will be performed in the near future in order to clarify their taxonomic status.

In conclusion, we have described the construction of *Leptospira* species-specific probes produced by using AP-PCR, in order to identify three of the main pathogenic species of *Leptospira*. These probes, which can be used in nonspecialized laboratories, will improve the identification of leptospires at the species level. This can be achieved within 2 days after isolation. This method will complement other molecular tools available for the identification of *Leptospira* isolates, allowing initial rapid identification of strains at the species level. It will contribute to the development of epidemiological studies needed to determine the ecological significance of these recently delimited species.

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