MOLECULAR DIAGNOSIS

Differentiation of *Leptospira* species and serovars by PCR-restriction endonuclease analysis, arbitrarily primed PCR and low-stringency PCR

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Reference strains from 30 serovars representing seven species of *Leptospira* and 48 recent isolates from human patients, dogs and rats, were characterised by polymerase chain reaction-restriction endonuclease analysis (PCR-REA), arbitrarily primed PCR (AP-PCR) and low stringency PCR (LS-PCR). PCR-REA analysis yielded seven groups among 29 serovars of pathogenic *Leptospira*; the non-pathogenic *L. biflexa* serovar *patoc* was not amplified with the primer pairs studied. AP-PCR and LS-PCR fingerprinting resulted in 25 and 21 distinct profiles, respectively, among the 30 reference strains. The results of the three PCR-based techniques were highly concordant and were in general agreement with those from previous DNA studies, confirming the high level of polymorphism among *Leptospira* species and serovars, and supported the concept of the serovar as the basic taxonomic unit of leptospiral classification. Results of the PCR-based typing methods for 11 randomised leptospiral strains, 36 clinical isolates from human patients and dogs and 12 survey isolates from trapped rats agreed with those from serological identification. With one exception, isolates of the same serovar gave identical profiles irrespective of the source. AP-PCR and LS-PCR are simple to perform and interpret, and appear to be useful for characterising isolates of *Leptospira* spp. for diagnostic and epidemiological purposes.

Introduction

Leptospirosis is an ubiquitous zoonotic infection, the spectrum of which ranges from subclinical infection to the severe, multi-organ syndrome characterised by jaundice and renal failure and known as Weil's disease. Pathogenic leptospires of the species *Leptospira interrogans sensu lato* were traditionally classified into 23 serogroups, each comprising several serologically related serovars [1, 2]. Currently, over 200 pathogenic serovars have been described [2]. Non-pathogenic serovars were similarly classified into a single species, *L. biflexa*. However, the phenotypic classification has been replaced by a genomic one in which 11 species are defined, based upon DNA relatedness [3, 4]. Pathogenic serovars are found within eight of these genomic species of *Leptospira*.

The precise identification and classification of *Leptospira* spp. is important for epidemiological and public

health surveillance, as different serovars can exhibit different host specificities and may not be associated with a particular clinical form of infection [5, 6]. Serological detection of antibodies to individual serogroups requires the use of the microscopic agglutination test (MAT), the interpretation of which is complex [7]. Although the MAT detects antibodies against specific serovars, cross-reactions occur between related serovars and hence the results of the MAT can be interpreted only as serogroup-specific (e.g., the serogroup Icterohaemorrhagiae rather than the serovar *copenhageni*). In addition, the use of the MAT to diagnose infection requires paired sera to allow the detection of rising titres. Paradoxical and anamnestic responses complicate interpretation further. However, the test remains important because of the epidemiological value of knowing presumptively which serogroup is involved in an infection.

On the island of Barbados, about 97% of clinical leptospiral infections are caused by three serogroups of leptospires. Serogroups Autumnalis, Icterohaemorrhagiae and Ballum account for 60%, 23% and 14% of infections, respectively [8]. Only four serovars have been isolated in Barbados despite intensive investiga-

tion for over 20 years: *bim* (serogroup Autumnalis), *copenhageni* (serogroup Icterohaemorrhagiae), *arborea* (serogroup Ballum) and *bajan* (serogroup Australis) [9]. The clinical syndrome caused by each of these four serovars in Barbados is identical [9].

Several molecular techniques have been applied successfully to the subtyping of leptospiral strains [10–16] but these are not ideally suited to diagnostic laboratories, being complex and time-consuming. Recently, a new procedure for rapid differentiation of micro-organisms known as random amplified polymorphic DNA (RAPD) or arbitrarily primed polymerase chain reaction (AP-PCR), was described [17, 18]. This technique is based on the use of a single arbitrary primer in a PCR of low stringency to amplify segments of the genome. Highly diverse DNA banding patterns can be generated, which may allow discrimination between and within species [19–22]. The development of a PCR-based typing system for *Leptospira* spp. would be a considerable advance because of its requirement for very small quantities of DNA and its simplicity.

In this study, PCR followed by restriction endonuclease analysis of the amplified target (PCR-REA), AP-PCR, and low stringency PCR (LS-PCR) with a pair of specific primers, were used to characterise *Leptospira* reference serovars and then to type leptospires isolated from recent specimens. LS-PCR was studied because it combined the principle of using

an arbitrary primer at low stringency with the specificity of 'diagnostic' primers [23]. The potential of the methods to identify pathogenic *Leptospira* isolates was also assessed with several randomised leptospiral strains.

Materials and methods

Leptospiral strains and culture conditions

The 30 reference leptospiral serovars used in this study (Table 1) included those which are detected in the Caribbean region, together with serovars that are of ubiquitous distribution [2]. Cultures were derived from the collection of the Barbados Leptospira Laboratory; 11 of these were randomised, coded and investigated blind by each method. Twenty-seven isolates from patients with acute leptospirosis in Barbados between July 1990 and October 1993, nine isolates from four sick dogs that were euthanised between December 1992 and September 1993, and 12 from rats trapped between September 1994 and April 1995 (Table 2) were also studied. Leptospires were maintained in semi-solid or liquid EMJH medium (Difco, USA), supplemented with 5-fluorouracil [24, 25].

DNA isolation and purification

Genomic DNA was isolated and purified from 7-day cultures of all *Leptospira* isolates by methods described previously [26], followed by treatment with RNAase A

Table 1. *Leptospira* reference serovars used in this study

Species	Serogroup	Serovar	Strain
<i>L. interrogans</i>	Australis	<i>bratislava</i>	Jez Bratislava
<i>L. interrogans</i>	Bataviae	<i>bataviae</i>	Swart
<i>L. interrogans</i>	Canicola	<i>canicola</i>	Hond Utrecht IV
<i>L. interrogans</i>	Canicola	<i>portlandvere</i>	MY 1039
<i>L. interrogans</i>	Hebdomadis	<i>kremastos</i>	Kremastos
<i>L. interrogans</i>	Icterohaemorrhagiae	<i>copenhageni</i>	M20
<i>L. interrogans</i>	Icterohaemorrhagiae	<i>icterohaemorrhagiae</i>	RGA
<i>L. interrogans</i>	Louisiana	<i>lanka</i>	R 740
<i>L. interrogans</i>	Pomona	<i>pomona</i>	Pomona
<i>L. interrogans</i>	Pyrogenes	<i>pyrogenes</i>	Salinem
<i>L. interrogans</i>	Sejroe	<i>hardjo</i>	Hardjoprajitno
<i>L. borgpetersenii</i>	Ballum	<i>arborea</i>	Arborea
<i>L. borgpetersenii</i>	Ballum	<i>ballum</i>	Mus 127
<i>L. borgpetersenii</i>	Sejroe	<i>sejroe</i>	M 84
<i>L. borgpetersenii</i>	Tarassovi	<i>tarassovi</i>	Perepelitsin
<i>L. noguchii</i>	Autumnalis	<i>fortbragg</i>	Fort Bragg
<i>L. noguchii</i>	Louisiana	<i>louisiana</i>	LSU 1945
<i>L. noguchii</i>	Louisiana	<i>orleans</i>	LSU 2580
<i>L. noguchii</i>	Panama	<i>panama</i>	CZ 214
<i>L. santarosai</i>	Bataviae	<i>brasiliensis</i>	An 776
<i>L. santarosai</i>	Mini	<i>georgia</i>	LT 117
<i>L. santarosai</i>	Sejroe	<i>trinidad</i>	TRVL 34056
<i>L. santarosai</i>	Sejroe	<i>caribe</i>	TRVL 61866
<i>L. kirschneri</i>	Autumnalis	<i>bim</i>	1051
<i>L. kirschneri</i>	Cynopteri	<i>cynopteri</i>	3522 C
<i>L. kirschneri</i>	Grippotyphosa	<i>grippotyphosa</i>	Moskva V
<i>L. biflexa</i>	Semarang	<i>patoc</i>	Patoc I
* <i>L. inadai</i>	Panama	<i>mangus</i>	TRVL 137774
* <i>L. noguchii</i>	Australis	<i>bajan</i>	Toad 60
* <i>L. noguchii</i>	Australis	<i>barbadensis</i>	Toad 67

*New species designations (A.Kaufmann, personal communication).

Table 2. Summary of the *Leptospira* isolates used in this study

Strain	Source	Serovar	Strain	Source	Serovar
P692	blood, human	<i>arborea</i> ^a	D752b	kidney, dog	<i>copenhageni</i>
RE06	kidney, rat	<i>arborea</i>	D756	kidney, dog	<i>copenhageni</i>
RE07	kidney, rat	<i>arborea</i>	P678	blood, human	<i>bim</i> ^b
RE09	kidney, rat	<i>arborea</i>	P687	blood, human	<i>bim</i>
RK02	kidney, rat	<i>arborea</i>	P710	blood, human	<i>bim</i>
RM02	kidney, rat	<i>arborea</i>	P714	blood, human	<i>bim</i>
P703	blood, human	<i>bajan</i> ^c	P715	blood, human	<i>bim</i>
P786	blood, human	<i>copenhageni</i> ^d	P721	urine, human	<i>bim</i>
P796	blood, human	<i>copenhageni</i>	P739	blood, human	<i>bim</i>
P808	urine, human	<i>copenhageni</i>	P746	blood, human	<i>bim</i>
P824	blood, human	<i>copenhageni</i>	P747	blood, human	<i>bim</i>
P895a	aqueous humour, human	<i>copenhageni</i>	P788	urine, human	<i>bim</i>
P895b	CSF, human	<i>copenhageni</i>	P822	blood, human	<i>bim</i>
RE11	kidney, rat	<i>copenhageni</i>	P823	blood, human	<i>bim</i>
RF03	kidney, rat	<i>copenhageni</i>	P826	blood, human	<i>bim</i>
RG01	blood, rat	<i>copenhageni</i>	P842	blood, human	<i>bim</i>
RH06	kidney, rat	<i>copenhageni</i>	P843	urine, human	<i>bim</i>
RJ01	kidney, rat	<i>copenhageni</i>	P851	blood, human	<i>bim</i>
RJ08	kidney, rat	<i>copenhageni</i>	P877	urine, human	<i>bim</i>
RK04	kidney, rat	<i>copenhageni</i>	P883	blood, human	<i>bim</i>
D744	blood, dog	<i>copenhageni</i>	P937	blood, human	<i>bim</i>
D745a	blood, dog	<i>copenhageni</i>	D762a	cerebellum, dog	<i>bim</i>
D745b	kidney, dog	<i>copenhageni</i>	D762b	medulla oblongata, dog	<i>bim</i>
D752a	aqueous humour, dog	<i>copenhageni</i>	D762c	kidney, dog	<i>bim</i>

^a*L. borgpetersenii* serovar *arborea*; ^b*L. kirschneri* serovar *bim*; ^c*Leptospira noguchii* serovar *bajan*; ^d*L. interrogans* serovar *copenhageni*.

for 30 min at 37°C. Extracts were diluted 10-fold with sterile distilled water before amplification. Crude DNA samples were prepared by lysis of cultures, previously diluted 10-fold in sterile distilled water, at 100°C (in a vigorously boiling waterbath) for 15 min, followed by rapid chilling on ice. Samples were then centrifuged for 10 s at 13 000 *g* and the supernates were stored at -20°C for up to 1 week before analysis.

PCR-restriction endonuclease analysis

PCR was performed in a total volume of 50 µl with primer pairs G1/G2 or B64-I/B64-II [23] as described previously [26]. Controls containing sterile distilled water instead of template material were included with every experiment. After amplification, a 5-µl volume of each sample was electrophoresed in agarose 2% w/v gels in Tris-borate-EDTA (TBE) buffer containing ethidium bromide 0.5 µg/ml.

Samples (10 µl) of the PCR products were digested with restriction endonucleases in a final volume of 20 µl, containing 1 µl enzyme and 2 µl of the appropriate buffer, according to the instructions of the suppliers (Boehringer Mannheim, Germany or New England Biolabs, USA). Products amplified with primers G1/G2 were digested separately with *Dde*I and *Nde*II at 37°C, and *Mae*II at 50°C, while products resulting from amplification with primers B64-I/B64-II were digested separately with *Aci*I, *Mnl*I, *Nla*III and *Hinf*I at 37°C. Restriction endonucleases *Dde*I, *Nde*II, *Mae*II, and *Hinf*I were purchased from Boehringer Mannheim, and *Aci*I, *Mnl*I and *Nla*III were obtained from New England Biolabs, USA. All

digestions were carried out for 3 h. Restriction endonucleases were selected on the basis of the published sequences of the amplified products and the expected variation within these sequences [23] with the program DNASIS (Pharmacia LKB, Uppsala, Sweden). The resulting DNA fragments were separated by electrophoresis in agarose 2% w/v gels in Tris-acetate-EDTA buffer (TAE) containing ethidium bromide 0.5 µg/ml. TAE buffer was used instead of TBE because it was found to give better resolution of restriction fragments.

Arbitrarily-primed PCR

In preliminary experiments, eight arbitrary primers (purchased from Eurogenetic, Belgium), designed with sequences that were unlikely to self-prime or form hairpin structures and with varied GC content (40–80%), were investigated for AP-PCR analysis of multiple isolates of serovars *copenhageni* and *bim* (data not shown). Based on the fingerprint clarity and discrimination obtained, primer PB-1 (5'-GCG CTG GCT CAG) was used for AP-PCR analysis of all 89 *Leptospira* strains, including the 11 randomised strains. PCR mixtures (final volume 50 µl) contained 50 ng of purified DNA or 40 µl of the rapid-lysis preparation, 0.3 µM primer, 250 µM of each dNTP, 3 mM MgCl₂, 0.5 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus) in 10 mM Tris-HCl (pH 9.0) and 50 mM KCl. Controls were used as described above for PCR-REA. Reaction mixtures were overlaid with 100 µl of mineral oil and amplified in a Pharmacia LKB Gene ATAQ controller. The temperature programme for amplification consisted of one cycle of 7 min at 94°C, 1 min at

40°C and 1 min at 72°C; four cycles of 1 min at 94°C, 1 min at 40°C and 1 min at 72°C; 24 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and one cycle of 1 min at 94°C, 1 min at 55°C and 7 min at 72°C. Reaction products (15- μ l samples) were electrophoresed in agarose 2% w/v gels in TBE containing ethidium bromide 0.5 μ g/ml. The sensitivity of AP-PCR was tested with amounts of DNA extracted from serovar *copenhageni* M20 ranging from 10 ng to 50 fg, in the amplification method described above.

Low stringency PCR

DNA extracted from the 89 strains was amplified with primers G1/G2. The components of the reaction mixtures were in a total volume of 50 μ l as described previously [26]. Mixtures were denatured initially at 95°C for 5 min and then subjected to amplification comprising five cycles of 1 min at 95°C, 1 min at 35°C, and 1 min at 72°C, followed by 24 cycles of 1 min at 94°C, 1 min at 40°C, and 1 min at 72°C, and a final cycle of 1 min at 94°C, 1 min at 40°C and 7 min at 72°C to ensure complete extension. The amplified products were analysed on agarose 2% w/v gels in TBE buffer containing ethidium bromide 0.5 μ g/ml. The sensitivity of LS-PCR was evaluated with amounts of DNA extracted from serovar *arborea* Arborea ranging from 10 ng to 50 fg in the amplification method described above.

Reproducibility and discriminatory index

The reproducibility and stability of PCR-REA, AP-PCR and LS-PCR fingerprints were determined by amplifications on numerous occasions. Each isolate was extracted five times and amplified at least three times from each extraction. The discriminatory index of the methods, i.e., the probability of two unrelated strains being characterised as the same type, was calculated from the equation derived by Hunter and Gaston [27].

Results

Comparison of template preparation conditions

Strains of serovars *copenhageni* and *bim* were used to optimise the conditions for the PCR-based characterisation methods. The results from DNA prepared by crude lysis of cultures by boiling for 15 min were found to be similar to those from purified DNA. Reproducible PCR results were obtained with purified DNA samples treated with RNAase A; RNAase treatment did not influence the amplification of DNA obtained by crude lysis of leptospiral cells. For all subsequent experiments, either crude lysed DNA or purified DNA treated with RNAase A was used.

PCR-REA analysis

Three, four and two polymorphic variations were found when the 285-bp fragment (generated by primers G1/G2) was digested with *Dde*I, *Nde*II and *Mae*II, respectively (Fig. 1). No polymorphism was noted for *L. kirschneri* serovars *bim* and *cynopteri* when primers B64-I/B64-II were used for amplification (not shown). The predicted fragment sizes resulting from digestion of this 563-bp amplicon with *Aci*I were 167 and 396 bp, with *Mn*II 120, 193 and 252 bp, with *Nla*III 104, 184 and 275 bp, and with *Hin*fl 6, 61, 84, 101, 121 and 187 bp. In each case, either two or three bands could be distinguished on agarose gel electrophoresis (for *Hin*fl, only two bands of 101/121 and 187 bp could be distinguished). *L. biflexa* serovar *patoc* was not amplified by either primer pair.

Seven groups were identified on the basis of PCR-REA patterns (Table 3). Group I consisted of all serovars from *L. borgpetersenii* and *L. santarosai*, and *L. interrogans* serovar *kremastos*. Group II contained 10 of the 11 serovars from *L. interrogans*, and *L. kirschneri* serovar *grippotyphosa*. Serovars *fortbragg* (*L. noguchii*) and *mangus* (*L. inadai*) were designated

Fig. 1. Restriction profiles of PCR products from 27 *Leptospira* strains generated by primers G1/G2. **A**, Digestion with *Dde*I: lanes 2, 3, profile 1; 4, 5, profile 2; 6, 7, profile 3. **B**, Digestion with *Nde*II: lanes 8, 9, profile 1; 10, 11, profile 2; 12, 13, profile 3; 14, 15, profile 4. **C**, Digestion with *Mae*II: lane 16, profile 2; 17, profile 1. Lanes 1 and 18 contain DNA size marker VIII (Boehringer Mannheim).

Table 3. Results of PCR-REA, AP-PCR and LS-PCR for 30 reference serovars of *Leptospira* spp.

Species	Serovar	PCR-REA profile*	AP-PCR profile	LS-PCR profile
<i>L. borgpetersenii</i>	<i>arborea</i>	1-1-1	1	1
<i>L. borgpetersenii</i>	<i>ballum</i>	1-1-1	2	1
<i>L. borgpetersenii</i>	<i>sejroe</i>	1-1-1	3	2
<i>L. borgpetersenii</i>	<i>tarassovi</i>	1-1-1	4	3
<i>L. santarosai</i>	<i>brasiliensis</i>	1-1-1	5	4
<i>L. santarosai</i>	<i>caribe</i>	1-1-1	6	5
<i>L. santarosai</i>	<i>georgia</i>	1-1-1	7	6
<i>L. santarosai</i>	<i>trinidad</i>	1-1-1	8	7
<i>L. interrogans</i>	<i>kremastos</i>	1-1-1	9	8
<i>L. interrogans</i>	<i>bataviae</i>	2-2-1	10	9
<i>L. interrogans</i>	<i>bratislava</i>	2-2-1	11	10
<i>L. interrogans</i>	<i>canicola</i>	2-2-1	12	11
<i>L. interrogans</i>	<i>copenhageni</i>	2-2-1	13	11
<i>L. interrogans</i>	<i>hardjo</i>	2-2-1	14	11
<i>L. interrogans</i>	<i>icterohaemorrhagiae</i>	2-2-1	13	11
<i>L. interrogans</i>	<i>lanka</i>	2-2-1	15	11
<i>L. interrogans</i>	<i>pomona</i>	2-2-1	16	12
<i>L. interrogans</i>	<i>portlandvere</i>	2-2-1	12	11
<i>L. interrogans</i>	<i>pyrogenes</i>	2-2-1	10	13
<i>L. kirschneri</i>	<i>grippotyphosa</i>	2-2-1	17	14
<i>L. noguchii</i>	<i>fortbragg</i>	3-3-2	18	15
<i>L. inadai</i> †	<i>mangus</i>	3-3-2	19	15
<i>L. noguchii</i>	<i>panama</i>	1-2-1	7	6
<i>L. noguchii</i> ‡	<i>bajan</i>	1-4-1	20	16
<i>L. noguchii</i> ‡	<i>barbadensis</i>	1-4-1	20	16
<i>L. noguchii</i>	<i>louisiana</i>	3-3-1	21	17
<i>L. noguchii</i>	<i>orleans</i>	3-3-1	22	18
<i>L. kirschneri</i>	<i>bim</i>	1-1-1-1	23	19
<i>L. kirschneri</i>	<i>cynopteri</i>	1-1-1-1	24	20
<i>L. biflexa</i>	<i>patoc</i>	-‡	25	21

*PCR-REA profile numbers are derived from the restriction patterns of the 285-bp fragment generated with G1/G2 primers (except for *bim* and *cynopteri*) generated by *Dde*I, *Nde*II and *Mae*II, respectively (see Fig. 1). For *bim* and *cynopteri*, the numbers in the profiles refer to the 563-bp fragment amplified with B64-I/B64-II primers, after digestion with *Aci*I, *Mnl*I, *Nla*III and *Hinf*I, respectively.

†New species designations (A.Kaufmann, personal communication).

‡*L. biflexa* serovar *patoc* was not amplified with either primer pair.

group III, while group IV contained only *L. noguchii* serovar *panama*. Group V consisted of *L. noguchii* serovars *bajan* and *barbadensis*, and group VI contained *L. noguchii* serovars *louisiana* and *orleans*. *L. kirschneri* serovars *bim* and *cynopteri* were assigned to group VII.

AP-PCR analysis

AP-PCR fingerprints of leptospiral genomic DNA consisted of discrete and reproducible sets of products (Fig. 2). A total of 25 distinct profiles was identified among the 30 *Leptospira* serovars examined (Table 3). Serovars within a species had common bands in their profiles, while fingerprints differed markedly between serovars from different species. As observed with PCR-REA, serovars *copenhageni* and *icterohaemorrhagiae* were indistinguishable; as were serovars *canicola* and *portlandvere*; serovar *pyrogenes* and serovar *bataviae*; and serovars *bajan* and *barbadensis*. Serovars *georgia* and *panama* also produced indistinguishable profiles. Although there was no substantial difference between the patterns resulting from use of purified DNA as the target and those resulting from supernates of boiled organisms, some faint bands differed occasionally. Similarly, there was no difference in major bands produced from samples prepared and amplified on different occasions. All serovars, including *patoc*, had

one band (c. 320 bp in size) in common which provided an internal control for the strains used in the study. AP-PCR profiles were indistinguishable for 10–200 ng of target, although at lower concentrations some of the fainter bands were no longer visible (data not shown).

LS-PCR analysis

When primers G1/G2 were used for LS-PCR, the specific (diagnostic) product of 285 bp was evident as the most intense component of the 21 profiles from the 30 serovars, together with a fairly complex set of low stringency products (Fig. 3). The LS-PCR profiles were found to be serovar-specific in some cases (Table 3), but indistinguishable patterns were observed for the following: (i) serovars *ballum* and *arborea*; (ii) serovars *copenhageni*, *icterohaemorrhagiae*, *canicola*, *portlandvere*, *hardjo* and *lanka*; (iii) serovars *fortbragg* and *mangus*; (iv) serovars *georgia* and *panama*; and (v) serovars *bajan* and *barbadensis*. Some serovars closely related by serology yielded very different LS-PCR profiles, including (i) serovars *panama* and *mangus*; (ii) serovars *trinidad* and *caribe*; and (iii) serovars *louisiana* and *orleans*. Similar LS-PCR profiles were given by other closely related serovars but these could usually be distinguished on the basis of two or more bands. The same LS-PCR pattern was

Fig. 2. AP-PCR fingerprints of genomic DNA from 30 *Leptospira* strains produced with primer PB-1. **a:** lanes 1 and 17, size marker VIII (Boehringer Mannheim); 2, serovar *ballum*; 3, *arborea*; 4, *canicola*; 5, *portlandvere*; 6, *copenhageni*; 7, *icterohaemorrhagiae*; 8, *pyrogenes*; 9, *grippotyphosa*; 10, *bataviae*; 11, *brasiliensis*; 12, *fortbragg*; 13, *bim*; 14, *cynopteri*; 15, *pomona*; 16, *tarassovi*. **b:** lanes 1 and 17, size marker (as for panel a); 2, serovar *sejroe*; 3, *hardjo*; 4, *trinidad*; 5, *caribe*; 6, *kremastos*; 7, *georgia*; 8, *panama*; 9, *mangus*; 10, *bratislava*; 11, *bajan*; 12, *barbadensis*; 13, *lanka*; 14, *louisiana*; 15, *orleans*; 16, *patoc*.

Fig. 3. LS-PCR fingerprints of genomic DNA from 30 *Leptospira* strains produced with primers G1/G2. **a:** lanes 1 and 17, size marker VIII (Boehringer Mannheim); 2, serovar *ballum*; 3, *arborea*; 4, *canicola*; 5, *portlandvere*; 6, *copenhageni*; 7, *icterohaemorrhagiae*; 8, *pyrogenes*; 9, *grippotyphosa*; 10, *bataviae*; 11, *brasiliensis*; 12, *fortbragg*; 13, *bim*; 14, *cynopteri*; 15, *pomona*; 16, *tarassovi*. **b:** lanes 1 and 17, size marker (as for panel a); 2, serovar *sejroe*; 3, *hardjo*; 4, *trinidad*; 5, *caribe*; 6, *kremastos*; 7, *georgia*; 8, *panama*; 9, *mangus*; 10, *bratislava*; 11, *bajan*; 12, *barbadensis*; 13, *lanka*; 14, *louisiana*; 15, *orleans*; 16, *patoc*.

observed for 10–200 ng of DNA of serovar *arborea*, although at lower concentrations some of the fainter bands were no longer visible (data not shown).

Discriminatory index

The discriminatory indices of the three PCR-based methods for characterising the 30 reference serovars were 0.77 for PCR-REA analysis, 0.986 for AP-PCR analysis and 0.956 for LS-PCR analysis.

Identification of *Leptospira* serovars

To test the utility of the three techniques for the identification of leptospiral isolates, profiles obtained for 11 randomised *Leptospira* serovars (coded LL1–LL11) were compared with the profiles of the 30 reference serovars. The AP-PCR and LS-PCR profiles are shown in Fig. 4. Based on comparative analysis of the profiles, LL1 was identified as *ballum*; LL2, as *copenhageni* or *icterohaemorrhagiae*; LL3, as *arborea*; LL4, as *bim* or *cynopteri*; LL5, as *bajan* or *barbadensis*; LL6, as *bim* or *cynopteri*; LL7, as *fortbragg*; LL8, as *copenhageni* or *icterohaemorrhagiae*; LL9, as *copenhageni* or *icterohaemorrhagiae*; LL10, as *arborea*; and LL11, as *canicola* or *portlandvere*. After the code was broken, serovars LL2, LL8 and LL9 were confirmed as *copenhageni*, serovars LL4 and LL6 as *bim*, serovar LL5 as *bajan*, and serovar LL11 as *canicola*; the remaining serovars were also

identified correctly. PCR-REA (digestion with *Dde*I, *Nde*II and *Mae*II only) also yielded the results expected for these serovars.

As the goal of these experiments was to apply the techniques to the characterisation of clinical isolates, it was important to show that the results obtained with the reference strains were reproducible with clinical samples. For this purpose, 48 isolates previously identified with monoclonal antibodies were analysed by PCR-REA with the appropriate restriction endonucleases. Thirty-nine isolates from human patients and rats were all epidemiologically unrelated, while there were nine isolates from four dogs. Restriction profiles obtained for the isolates were indistinguishable from those of the respective reference strains. The isolates of *arborea* (6), *bajan* (1), *bim* (22), and *copenhageni* (19) yielded profiles identical to those obtained for *arborea* Arborea, *bajan* Toad 60, *bim* 1051, and *copenhageni* M20, respectively.

Direct detection and identification of leptospiral DNA was attempted on samples of tissue taken from patients who were positive by PCR with primers G1/G2 or B64-I/B64-II. Digestion of DNA amplified from the tissues, with the appropriate enzymes, resulted in restriction patterns identical to those obtained from pure cultures of *copenhageni* strain M20 or *bim* strain 1051, respectively (data not shown).

Except for one human *bim* isolate (P715), the 48 leptospiral isolates produced AP-PCR profiles identical to that of the respective reference serovar (data not shown). LS-PCR of DNA from the 48 leptospiral isolates yielded profiles identical to those of the reference serovars, except for *bim* isolate P715 (data not shown). The discriminatory indices for AP-PCR and LS-PCR analysis of the multiple, epidemiologically unrelated clinical isolates of *copenhageni* and *bim* were zero and 0.099, respectively.

Discussion

Thirty reference strains of *Leptospira* of different serovars, including those commonly detected by isolation or serology in the Caribbean region, were characterised by PCR followed by restriction endonuclease digestion, fingerprinting by arbitrarily primed PCR and fingerprinting by low-stringency PCR with diagnostic primers (either G1/G2 or B64-I/B64-II). The three methods provided results that were in general agreement with each other and with the species assignments based on DNA-DNA homology [3, 4].

The differences in PCR-REA, AP-PCR or LS-PCR profiles for serovars belonging to different species were usually considerable and presumably reflect a large degree of sequence divergence between species.

Fig. 4. **a**, AP-PCR and **B**, LS-PCR fingerprints of genomic DNA from 11 randomised *Leptospira* serovars. Lanes 1–11, serovars LL1–LL11, respectively (see text); 12, size marker VIII (Boehringer Mannheim.).

Conversely, closely related serovars within the same species (such as *L. interrogans* serovars *canicola* and *portlandvere*, or *L. interrogans* serovars *icterohaemorrhagiae* and *copenhageni*) were indistinguishable. Some closely related serovars, such as *L. borgpetersenii* serovars *arborea* and *ballum*, were differentiated only by AP-PCR. The data from the present study suggest that some of the serovars now grouped within a particular species would merit further study to determine their correct taxonomic assignments, for example, *L. kirschneri* serovar *grippotyphosa*. Serovar *grippotyphosa* was initially classified within the species *L. interrogans sensu stricto* [4], but was then reclassified within *L. kirschneri* [3]. PCR-REA data from this study show that serovar *grippotyphosa* has the same pattern as 10 other serovars of *L. interrogans*, while it does not amplify with the B64-I/B64-II primers designed for amplification of *L. kirschneri*. The data therefore support the initial placement of serovar *grippotyphosa* in *L. interrogans sensu stricto* [4].

Some serovars within different species, such as *L. noguchii* serovar *panama* and *L. santarosai* serovar *georgia*, were indistinguishable by AP-PCR and LS-PCR, but produced distinguishable PCR-REA patterns. The non-pathogenic serovar *patoc* produced LS-PCR and AP-PCR profiles very different from all other serovars tested, and did not amplify with either of the primer sets designed for detection of pathogenic serovars [23].

The results of PCR-REA analysis of the 285-bp product generated by G1/G2-primed amplification showed that there was reasonable agreement between profile and species. While the present study was in progress, reports were published of similar studies with PCR-REA [28] and LS-PCR [29]. Savio *et al.* [28] developed a PCR for amplification of a repetitive element present in serovars of *L. interrogans sensu stricto* and in two serovars of *L. borgpetersenii*. Restriction analysis of this product with either *DdeI* or *HinfI* produced results similar to those of the present study; most serovars could be differentiated but serovars *icterohaemorrhagiae* and *copenhageni* were indistinguishable. Field isolates of serovar *pomona* produced REA patterns identical to the reference strain. The LS-PCR described by de Cabellero *et al.* [29] used the same G1/G2 primer pair [23] and produced results similar to those reported in the present study. However, serovars *icterohaemorrhagiae* and *copenhageni* again gave identical profiles, emphasising the very close relationship between these two serovars. This is in agreement with the results from ribotyping [13] and chromosomal REA [30, 31].

When 48 clinical and survey isolates of serovars *arborea*, *bim* and *copenhageni* were examined in the present study, the AP-PCR and LS-PCR profiles were identical for isolates within each serovar, with the

exception of a single human isolate of *bim* which differed from all other isolates of this serovar. Multiple isolates of serovars *bim* and *copenhageni* from three dogs were studied. Isolates from each dog were indistinguishable from each other and from their respective reference strains. Although the discriminatory indices of AP-PCR and LS-PCR were very high for analysis of the reference serovars, indices of zero or very close to zero were obtained for the 48 isolates of *arborea*, *bim* and *copenhageni*. This could indicate that the methods either cannot discriminate between strains of the same serovar, or that the Barbados isolates of each serovar originated from a single clone and are genetically homogeneous. Therefore, further investigation is necessary to determine which of these explanations is more probable.

Because AP-PCR amplification utilises an arbitrarily chosen primer, and low stringency annealing conditions are employed for both AP-PCR and LS-PCR, the potential for variation in products is considerable. However, both approaches consistently resulted in reproducible patterns from either purified or crude DNA, which were unaffected by the amount of target DNA. Welsh and McClelland [17] reported similar results for AP-PCR, and rapid lysis preparations have been used successfully in PCR-based analysis of *Pseudomonas aeruginosa* [19] and *Listeria monocytogenes* [21]. PCR-based characterisation is ideal for the rapid identification of leptospiral isolates and has distinct advantages over REA of total genomic DNA [10, 15] or DNA-DNA hybridisation [11, 14], both of which require large amounts of purified DNA. Moreover, the PCR-derived profiles are less complex and, therefore, easier to compare than genomic REA profiles, and the techniques do not require high-resolution electrophoresis, unlike pulsed-field gel electrophoresis. These factors, and the slow growth rate of leptospires (especially from primary cultures), make PCR-based characterisation an attractive approach. Despite the fact that PCR-REA analysis targeted a relatively conserved region of the leptospiral genome [23], whereas AP-PCR and LS-PCR analysed more of the genome, this method showed good (but not absolute) agreement with species identification for the reference serovars examined in this study. Moreover, because culture of leptospires from clinical specimens is often unsuccessful, determination of restriction fragment length polymorphisms in PCR products obtained directly from clinical material may be the only data obtainable from such specimens. In cases where isolation is successful, the AP-PCR and LS-PCR methods can be employed for the identification and characterisation of the isolates for diagnostic and epidemiological purposes.

The results of this study showed that PCR-based fingerprinting facilitates characterisation at the inter- and intra-species level, and specifically, the identification of pathogenic *Leptospira* isolates to the serovar

level. The three PCR-based techniques were highly concordant and support the concept of the serovar as the basic taxonomic unit of leptospiral classification.

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