Low-stringency single specific primer PCR for identification of *Leptospira*

Marluce A. Assunção Oliveira,¹ Otávia L. Caballero,⁴ Annamaria R. Vago,² Rudy A. Harskeerl,⁵ Álvaro J. Romanha,⁶ Sérgio D. J. Pena,³ Andrew J. G. Simpson⁴ and Matilde Cota Koury¹

Correspondence

Matilde Cota Koury
kourymat@mono.icb.ufmg.br

^{1–3}Departamento de Microbiologia¹, Departamento de Morfologia² and Departamento de Bioquímica e Imunologia³, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, CP 486, CEP: 31270-901, Belo Horizonte, Minas Gerais, Brazil

⁴Laboratory of Cancer Genetics, Ludwig Institute for Cancer Research, São Paulo, Brazil

⁵Department of Biomedical Research, Royal Tropical Institute, Amsterdam, The Netherlands

⁶Centro de Pesquisas 'René Rachou' – FIOCRUZ, Belo Horizonte, Minas Gerais, Brazil

Thirty-five *Leptospira* serovars from the species *Leptospira interrogans*, *Leptospira borgpetersenii*, *Leptospira santarosai*, *Leptospira kirschneri*, *Leptospira weilii*, *Leptospira biflexa* and *Leptospira meyeri* were characterized by the low-stringency single specific primer PCR (LSSP-PCR) technique. LSSP-PCR analysis was performed to detect DNA polymorphisms in a 285 bp DNA fragment amplified from genomic DNA with G1 and G2 selected primers. Similar LSSP-PCR profiles were obtained for serovars from the same genomic species, while serovars from non-related species produced distinct multiband patterns. Based on the data from sequence analysis, all genomic fragments amplified with G1 and G2 primers from distinct serovars of *Leptospira* were 285 bp in length, with nucleotide variation observed most frequently among different genomic species. The simplicity and accuracy of the LSSP-PCR technique were found to be suitable for identification of *Leptospira* species.

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INTRODUCTION

Leptospirosis is an ubiquitous zoonotic infection. The genus *Leptospira* is classified in 13 genomic species based upon DNA relatedness: *Leptospira interrogans sensu stricto*, *Leptospira borgpetersenii*, *Leptospira inadai*, *Leptospira noguchi*, *Leptospira santarosai*, *Leptospira weilii*, *Leptospira biflexa sensu stricto*, *Leptospira meyeri*, *Leptospira wolbachii*, *Leptospira parva*, *Leptospira kirschneri*, *Leptospira alexanderi* and *Leptospira fainei* (Yasuda *et al.*, 1987). The precise identification and classification of leptospires is important for epidemiological and public health surveillance.

LSSP-PCR (low-stringency single specific primer PCR) is a rapid and simple technique that detects sequence variations in DNA fragments by amplification under very low-stringency conditions with a single primer specific for one of the extremities of the template (Pena *et al.*, 1994).

Molecular diagnostic methods are increasingly being used

for clinical diagnosis in endemic areas because of their sensitivity and specificity. PCR amplification techniques should help to characterize any *Leptospira* DNA sequences present. Especially in the early stage of an outbreak, it can be extremely valuable to characterize further any diagnostic DNA sequences that have been amplified in order to confirm the amplification as being definitively derived from *Leptospira* and not due to an anomalous amplification. This can be done by hybridization, restriction endonuclease digestion or DNA sequencing. Each of these approaches, however, requires the use of additional reagents and equipment and thus adds to the complexity of the diagnostic process.

In this context, LSSP-PCR represents an important alternative in that it involves a simple repetition of the PCR process with one of the two primers used in the initial amplifications, but it is highly sensitive to the sequence content of the gene fragment being analysed. Furthermore, variations in the sequence of the amplified product can assist in the precise identification of the infecting organism to the species and serovar levels. This is of critical value in the epidemiological assessment of an outbreak and in attempts to identify potential sources of exposure.

We have shown previously that preliminary *Leptospira*

Abbreviation: LSSP-PCR, low-stringency single specific primer-PCR.

An alignment of the sequences of PCR products obtained with primers G1 and G2 is available as supplementary material in JMM Online (<http://jmm.sgmjournals.org/>).

identification could be achieved on the basis of the apparent mobility of PCR products generated for *Leptospira* in polyacrylamide gels (Oliveira *et al.*, 1995). We have also demonstrated that reamplification of the same products by LSSP-PCR could be used to identify some *Leptospira* species (Oliveira *et al.*, 1994).

In this work, we have extended our initial study and performed LSSP-PCR for genetic characterization of 35 serovars belonging to seven genomic species of *Leptospira*. Specific banding profiles were obtained for serovars originating from the same genomic species. Thus, LSSP-PCR represents a new, sensitive and efficient tool for the molecular typing of serovars of *Leptospira*.

METHODS

Leptospira serovars. Thirty-five *Leptospira* serovars from the species *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. kirschneri*, *L. weilii*, *L. biflexa* and *L. meyeri* were included in this study (Table 1). The *Leptospira* serovars were cultured in a liquid medium of Ellinghausen and McCullough, as modified by Johnson & Harris (1967) (EMJH medium), under aerobic conditions at 28 °C in the absence of light.

DNA isolation. Approximately 10^6 – 10^8 cells from 1 ml of a 7-day culture were collected by centrifugation at 13 000 *g* prior to DNA preparation. Subsequently, cells were incubated overnight at 50 °C with 200 µl lysis buffer [50 mM Tris/HCl, pH 8.0; 50 mM EDTA; 100 mM NaCl; 1% (w/v) SDS] containing 100 µg proteinase K ml⁻¹. DNA was extracted with phenol/chloroform and precipitated with ethanol (Tamai *et al.*, 1988).

LSSP-PCR. The LSSP-PCR technique is a two-step procedure: the first step utilizes two primers to obtain PCR products, which are then used as templates in a second step of amplification using low-stringency conditions and a single primer.

Specific PCR was undertaken with the primers G1 (5'-CTG AATCGCTGTATAAAAGT-3') and G2 (5'-GGAAACAAATGGT CGGAAG-3'), derived from sequences obtained from a genomic library of *L. interrogans* serovar *icterohaemorrhagiae* strain RGA, which were described previously (Gravekamp *et al.*, 1993). There was no cross-reaction of the G1 and G2 primers when used with several other spirochaetes (i.e. *Borrelia burgdorferi* and *Treponema reuteri*), various other micro-organisms such as *Mycobacterium* spp., *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Salmonella* spp., *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica* and *Escherichia coli*, or human DNA (Gravekamp *et al.*, 1991). Amplification was carried out in a volume of 10 µl containing 5 ng genomic DNA, 1.5 mM MgCl₂, 200 µM each of the four dNTPs, 0.4 IU *Taq* DNA polymerase (Cenbiot) and 1 pmol of each primer in 10 mM Tris/HCl, pH 8.0, 50 mM KCl, under 20 µl mineral oil. After an initial denaturation step of 94 °C for 3 min, the specific PCR program consisted of 30 cycles of 94 °C for 30 s, 51 °C for 1 min and 72 °C for 1 min. The last cycle consisted of an extension step at 72 °C for 3 min.

Products obtained from the PCR step were run on a 1.5% ethidium-bromide-stained agarose gel, excised from the gel by aspiration and added to Eppendorf tubes containing 50 µl double-distilled sterile water. After heating at 95 °C to melt the agarose, 1 µl of eluate was taken as the template for the LSSP-PCR.

LSSP-PCR was also carried out under 20 µl mineral oil in a volume of 10 µl containing 1 µl DNA template, 1.5 mM MgCl₂, 200 µM each of the four dNTPs, 1.6 IU *Taq* DNA polymerase (Cenbiot) and 48 pmol of primer G1 or G2 in 10 mM Tris/HCl, pH 8.0, 50 mM KCl. After a

denaturation step at 94 °C for 6 min, the LSSP-PCR program consisted of 35 cycles of 94 °C for 1 min and 30 °C for 1 min (Pena *et al.*, 1994). Five microlitres of LSSP-PCR products were analysed by electrophoresis on 8% (w/v) polyacrylamide gels followed by silver staining (Sanguinetti *et al.*, 1994).

Sequencing of PCR products. DNA sequencing was performed on PCR products obtained by amplification with G1 and G2 primers from genomic DNA of eight serovars from four distinct genomic species of *Leptospira*: *L. interrogans* (serovars *icterohaemorrhagiae*, *australis* and *hardjo*), *L. borgpetersenii* (serovars *hardjobovis* and *ballum*), *L. weilii* (serovars *celledoni* and *coxi*) and *L. meyeri* (serovar *ranarum*). For cloning and sequencing procedures, kits supplied by Applied Biosystems were used according to the manufacturer's instructions. Nucleotide sequences were determined in an Automated DNA sequencer (Applied Biosystems) and analysed with the PC Gene software (release 6.6; Genofit).

RESULTS

LSSP-PCR analysis

A 285 bp fragment was specifically amplified from all serovars of *Leptospira* used in this study (Table 1) by PCR with the primers G1 and G2. This fragment was further characterized using LSSP-PCR with primer G1 or G2.

In order to determine the capacity of LSSP-PCR to detect possible polymorphisms present in the target fragment of 285 bp, we initially used primer G2 for analysis of the PCR products originating from different genomic species of the genus *Leptospira* (Fig. 1). Distinct and informative profiles presenting multiple bands were obtained for four serovars belonging to *L. interrogans*, *L. borgpetersenii*, *L. biflexa* and *L. weilii*. Genetic signatures obtained from *Leptospira* with primer G2 were made up of fragments ranging from 100 to 1400 bp, with some larger than the 285 bp PCR products used as template for the LSSP-PCR.

We subsequently used the LSSP-PCR technique to analyse different serovars from the same genomic species (*L. interrogans*), with the goal of determining the usefulness of genetic signatures in the identification of serovars belonging to the same genomic species.

Fig. 2 shows LSSP-PCR profiles obtained through the analysis of 19 serovars from the genomic species *L. interrogans* with the primer G1. Very similar genetic signatures were obtained for all serovars analysed, which were mainly composed of three fragments (Table 2). Serovars *canicola*, *pomona* and *hebdomadis* presented an additional band of approximately 267 bp (Fig. 2, arrows; Table 2).

Gene signatures obtained through the analysis of 13 serovars belonging to the genomic species *L. interrogans* with the primer G2 are shown in Fig. 3. The main constitutive fragments of LSSP-PCR profiles are listed in Table 2. More complex, informative and identical profiles were obtained for each of the serovars analysed, with no distinction between the profiles obtained for the serovars *canicola*, *pomona* and *hebdomadis*.

In order to evaluate the potential usefulness of genetic

Table 1. *Leptospira* strains used in this study

The sizes of PCR products reflect their electrophoretic mobility (in bp) relative to DNA size standards; all products were shown by DNA sequencing to be 285 bp long.

Strain	Serogroup*	Serovar	PCR product (bp)
<i>L. interrogans</i>			
Ballico†	Australis ^{a,b,c}	<i>australis</i>	295
Akiyami A	Autumnalis ^{a,b}	<i>autumnalis</i>	295
Swart	Bataviae ^c	<i>bataviae</i>	295
Van Tienen	Bataviae ^{a,b,c}	<i>bataviae</i>	295
Hond Utrecht IV	Canicola ^{a,b}	<i>canicola</i>	295
Sentot	Djasiman ^{a,b}	<i>sentot</i>	295
Wijnberg	Icterohaemorrhagiae ^c	<i>copenhageni</i>	295
M 20	Icterohaemorrhagiae ^a	<i>copenhageni</i>	295
RGAT†	Icterohaemorrhagiae ^{a,b,c}	<i>icterohaemorrhagiae</i>	295
Naam	Icterohaemorrhagiae ^a	<i>naam</i>	295
Pomona	Pomona ^{a,b}	<i>pomona</i>	295
Salinem	Pyrogenes ^{a,b}	<i>pyrogenes</i>	295
Hardjoprajitno	Sejroe ^{a,c}	<i>hardjo</i>	295
Hebdomadis	Hebdomadis ^a	<i>hebdomadis</i>	295
Lai	Icterohaemorrhagiae ^c	<i>lai</i>	295
Sumner	Canicola ^a	<i>sumneri</i>	295
Vleermnis	Canicola ^a	<i>shueffneri</i>	295
Jalna	Australis ^c	<i>jalna</i>	295
BD 92	Australis ^c	<i>bangkok</i>	295
Muenchen C 90	Australis ^c	<i>muenchen</i>	295
Lora	Australis ^c	<i>lora</i>	295
<i>L. borgpetersenii</i>			
Castellon 3	Ballum ^{a,b,c}	<i>castellonis</i>	260
Veldrat Bataviae 46 ^T	Javanica ^{a,b}	<i>javanica</i>	255
Mus 127†	Ballum ^c	<i>ballum</i>	270
Lely 607†	Sejroe	<i>hardjobovis</i>	270
<i>L. santarosai</i>			
LT 821 ^T	Shermani ^{a,b}	<i>shermani</i>	255
CZ 390	Sarmin ^c	<i>weaveri</i>	280
TRVL 112499	Pyrogenes ^c	<i>princestown</i>	270
LT 117	Mini ^c	<i>georgia</i>	270
<i>L. kirschneri</i>			
Ndahambukuje	Icterohaemorrhagiae ^a	<i>ndahambukuje</i>	280
Moskva V	Grippotyphosa	<i>grippotyphosa</i>	280
<i>L. weilii</i>			
Celledoni ^T †	Celledoni ^c	<i>celledoni</i>	260
Coxi†	Javanica ^c	<i>coxi</i>	260
<i>L. biflexa</i>			
CH 11	Andamana ^a	<i>andamana</i>	270
Patoc I ^T	Semaranga ^a	<i>patoc</i>	270
<i>L. meyeri</i>			
Iowa City Frog ^T (ICF ^T)†	Ranarum ^c	<i>ranarum</i>	270
Veldrat Sem 173	Semaranga ^c	<i>semaranga</i>	270

*Serovars obtained from: *a*, Instituto Adolfo Lutz, São Paulo, Brazil; *b*, Laboratório de Leptospirose, FIOCRUZ, Rio de Janeiro, Brazil; *c*, Royal Tropical Institute, Amsterdam, The Netherlands.

†Strain used for DNA sequencing.

Fig. 1. LSSP-PCR profiles from different species of *Leptospira* with primer G2 in duplicate. Lanes: 1, molecular size marker (ϕ 174/*Hae*III); 2 and 3, *L. interrogans* serovar *canicola*; 4 and 5, *L. borgpetersenii* serovar *castellanis*; 6 and 7, *L. biflexa* serovar *andamana*; 8 and 9, *L. weilii* serovar *celledoni*. Reaction products were separated on an 8 % (w/v) polyacrylamide gel and silver-stained, as described in Methods.

signatures obtained by LSSP-PCR for genetic identification of serovars of *Leptospira*, we analysed serovars belonging to six different genomic species. Fig. 4 shows genetic signatures obtained with primer G1 for analysis of 15 distinct serovars: three from the species *L. borgpetersenii* (lanes labelled A), four from *L. santarosai* (B), two from *L. weilii* (C), two from *L. kirschneri* (D), two from *L. biflexa* (E) and two from *L. meyeri* (F). The main constitutive fragments of LSSP-PCR profiles are listed in Table 2.

In comparison with the signatures obtained with primer G1 with *L. interrogans* serovars (Fig. 2), all LSSP-PCR profiles obtained for the six different genomic species analysed (Fig.

4) were distinct from those obtained for *L. interrogans*, except for the LSSP-PCR profile obtained for the serovar *grippotyphosa* from *L. kirschneri*, which showed two fragments identical in size (180 and 270 bp) to those observed for the profiles of serovars from *L. interrogans* with primer G1 (Fig. 2, lanes 1–21; Fig. 4, lane 12). In general, all genomic species analysed with primer G1 displayed fragments that produced distinct genetic signatures for each group of analysed species, the similarity of which made it possible to assign the serovars within to a specific group (Fig. 4).

Specific genetic signatures were also obtained with primer G2 (Fig. 5) for serovars belonging to the same genomic species of *Leptospira*. LSSP-PCR profiles generated by primer G2 were mainly composed of fragments ranging from approximately 100 to 1400 bp. The major bands of the LSSP-PCR patterns are shown in Table 2.

Although the LSSP-PCR profiles obtained with primer G2 were more informative than those obtained with the same samples using primer G1, smaller differences were observed among serovars from different genomic species (Fig. 4). Nevertheless, specific fragments could be observed in profiles of serovars belonging to the same genomic species and therefore permit their identification within the reference group, especially in the genomic species *L. borgpetersenii* (Fig. 5, lanes labelled A), *L. santarosai* (B) and *L. biflexa* (E).

In general, genomic signatures obtained with primers G1 and G2 (Figs 4 and 5; Table 2) showed that serovars belonging to the same genomic species produced similar LSSP-PCR profiles, suggesting limited intra-specific polymorphism in the amplified genomic fragments, with more homogeneous profiles obtained for the genomic species *L. interrogans* (Figs 2 and 3; Table 2).

The stability and reproducibility of genetic signatures of *Leptospira* were confirmed by experiments in which we tested

Fig. 2. LSSP-PCR profiles of *L. interrogans* serovars with primer G1. Lanes: 1, molecular size marker (ϕ 174/*Hae*III); 2–22, *L. interrogans* serovars *australis* (lane 2), *autumnalis* (3), *bataviae* (strain Van Tienen) (4), *canicola* (5), *sentot* (6), *copenhageni* (strain M20) (7), *icterohaemorrhagiae* (8), *naam* (9), *pomona* (10), *pyrogenes* (11), *hardjo* (12), *hebdomadis* (13), *lai* (14), *bataviae* (strain Swart) (15), *lora* (16), *jalna* (17), *bangkok* (18), *muenchen* (19), *copenhageni* (strain Wijnberg) (20), *sumneri* (21) and *shueffneri* (22); 23, LSSP-PCR negative control.

Table 2. Molecular sizes of DNA fragments obtained by LSSP-PCR with G1 and G2 primers

Sizes of main fragments observed with each primer are given in bp. ND, Not determined.

Serovar(s)	G1	G2
<i>L. interrogans</i>		
<i>australis</i> , <i>autumnalis</i> , <i>bataviae</i> (strain Van Tienen), <i>sentot</i> , <i>copenhageni</i> (strain M20), <i>icterohaemorrhagiae</i> , <i>naam</i> , <i>pyrogenes</i> , <i>hardjo</i> , <i>lai</i> ,	180, 270, 310	110, 250, 280, 400, 500, 600
<i>bataviae</i> (strain Swart), <i>lora</i> , <i>jalna</i> , <i>bangkok</i> , <i>muenchen</i> , <i>copenhageni</i> (strain Wijnberg), <i>sumneri</i> , <i>shueffneri</i>	180, 270, 310	ND
<i>canicola</i> , <i>pomona</i> , <i>hebdomadis</i>	180, 270, 310, 267	110, 250, 280, 400, 500, 600
<i>L. borgpetersenii</i> serovars <i>castellonis</i> , <i>javanica</i> , <i>ballum</i>	190, 267, 400, 872	100, 150, 267, 275
<i>L. santarosai</i>		
<i>shermani</i> , <i>weaveri</i>	185, 267	150, 195, 275
<i>princestown</i> , <i>georgia</i>	185, 267	ND
<i>L. weilii</i> serovars <i>celledoni</i> , <i>coxi</i>	185, 234, 267, 400	150, 200, 275, 300
<i>L. kirschneri</i>		
<i>ndahambukuje</i>	180, 240, 265, 281, 500	150, 250, 300, 310, 320, 605
<i>grippotyphosa</i>	180, 270	ND
<i>L. biflexa</i> serovars <i>andamana</i> , <i>patoc</i>	180, 234, 267, 500	200, 250, 275, 300, 350
<i>L. meyeri</i> serovars <i>ranarum</i> , <i>semaranga</i>	185, 234, 267, 310	ND

Fig. 3. LSSP-PCR profiles of *L. interrogans* with primer G2. Lanes: 1, molecular size marker (ϕ 174/*Hae*III); 2–14, *L. interrogans* serovars *australis* (2), *autumnalis* (3), *bataviae* (strain Van Tienen) (4), *canicola* (5), *sentot* (6), *copenhageni* (strain M20) (7), *icterohaemorrhagiae* (8), *naam* (9), *pomona* (10), *pyrogenes* (11), *hardjo* (12), *hebdomadis* (13) and *lai* (14).

different amounts of DNA template (1 and 2 ng) with five distinct serovars from four species of the genus *Leptospira*, *L. interrogans*, *L. borgpetersenii*, *L. biflexa* and *L. kirschneri*. Identical LSSP-PCR profiles were obtained with primer G2 for each of the serovars analysed, even when distinct concentrations of DNA template were used (data not shown).

A comparison among genetic signatures obtained with primer G2 in separate experiments for *L. interrogans* serovars *canicola* (Fig. 1, lanes 2 and 3), *australis* and *naam* (data not shown) with other serovars belonging to this genomic species (Fig. 3, lanes 2–14) indicated a strong similarity between the profiles and reinforces the usefulness of genetic signatures obtained by LSSP-PCR for the identification of serovars belonging to the same genomic species of *Leptospira*.

In the same way, serovars *castellonis* (Fig. 1, lanes 4 and 5) and *javanica* (data not shown), belonging to the genomic species *L. borgpetersenii*, presented identical genetic signatures even when experiments were carried out separately and thus allowed the classification of the two serovars to the same group. Identical LSSP-PCR profiles were also obtained for serovars *andamana* (Fig. 1, lanes 6 and 7) and *patoc* (data not shown), both belonging to the genomic species *L. biflexa*.

DNA sequencing

In this study, we have undertaken the sequence analysis of genomic fragments amplified with primers G1 and G2 originated from genomic DNA of eight serovars belonging to four genomic species of *Leptospira*: *L. interrogans* (serovars *icterohaemorrhagiae*, *australis* and *hardjo*), *L. borgpetersenii* (serovars *hardjobovis* and *ballum*), *L. weilii* (serovars *celledoni* and *coxi*) and *L. meyeri* (serovar *ranarum*).

All sequenced fragments originating from different serovars were of the same size (285 bp). An alignment of the sequences obtained is available as supplementary material in JMM Online (<http://jmm.sgmjournals.org/>). Sequence analysis indicated the presence of three nucleotide alterations, at

Fig. 4. LSSP-PCR profiles from different species of *Leptospira* obtained using primer G1. Lanes: 1, molecular size marker (ϕ 174/*Hae*III); 2–4, *L. borgpetersenii* serovars *castellanis* (2), *javanica* (3) and *ballum* (4); 5–8, *L. santarosai* serovars *shermani* (5), *weaveri* (6), *princestown* (7) and *georgia* (8); 9 and 10, *L. weilii* serovars *celledoni* (9) and *coxi* (10); 11 and 12, *L. kirschneri* serovars *ndahambukuje* (11) and *grippotyphosa* (12); 13 and 14, *L. biflexa* serovars *andamana* (13) and *patoc* (14); 15 and 16, *L. meyeri* serovars *ranarum* (15) and *semaranga* (16).

Fig. 5. LSSP-PCR profiles from different species of *Leptospira* obtained using primer G2. Lanes: 1, molecular size marker (ϕ 174/*Hae*III); 2–4, *L. borgpetersenii* serovars *castellanis* (2), *javanica* (3) and *ballum* (4); 5 and 6, *L. santarosai* serovars *shermani* (5) and *weaveri* (6); 7 and 8, *L. weilii* serovars *celledoni* (7) and *coxi* (8); 9, *L. kirschneri* serovar *ndahambukuje*; 10 and 11, *L. biflexa* serovars *andamana* (10) and *patoc* (11).

positions 25, 90 and 217, between the serovars *icterohaemorrhagiae*, *australis* and *hardjo*, belonging to the genomic species *L. interrogans*; nine alterations, at positions 28, 31, 33, 34, 73, 100, 193, 217 and 220, between the serovars *hardjobovis* and *ballum* belonging to the species *L. borgpetersenii* and three nucleotide changes, at positions 139, 220 and 241, in the serovars *celledoni* and *coxi*, belonging to the genomic species *L. weilii*. The species *L. meyeri* was repre-

sented by a single serovar (*ranarum*) whose sequence, in comparison with that of *L. interrogans* serovar *icterohaemorrhagiae* (consensus), demonstrated the presence of 35 nucleotide substitutions, followed by *L. borgpetersenii*, with 43 changes, and *L. weilii*, with 50 changes.

Data obtained from the DNA sequence analysis indicated that there is greater variation in the sequence of amplified fragments obtained with primers G1 and G2 among different species and less variation in the sequence of these fragments within the same genomic species.

DISCUSSION

In the present study, we have undertaken the identification of 35 serovars belonging to seven genomic species of *Leptospira* through the analysis of a genomic fragment of 285 bp previously amplified by PCR. There was no cross-reaction of primers G1 and G2 with other micro-organisms or human DNA (Gravekamp *et al.*, 1991).

All serovars used in this study were submitted to LSSP-PCR using primer G1 or G2, both of which were capable of producing genetic signatures composed of multiple fragments. The profiles obtained with the primer G2 were more informative (Figs 1, 3 and 5).

The exact mechanism by which LSSP-PCR works is not well understood. One possibility is that the production of multiple fragments is related to the specificity of annealing of the primer to its complementary extremity and, with less specificity, to various sites present in the interior of the fragment. This is achieved in the reaction by the use of high concentrations of the single initiator and *Taq* DNA polymerase, with an extremely low annealing temperature. The extension of the complex initiator–template DNA by the *Taq* DNA polymerase produces a pattern of fragments of variable sizes, which can be explained through the dynamic reaction between the primer and its sites of annealing (specific or not)

as well as between the products generated at the beginning of the reaction, which can react as drivers. When the products of LSSP-PCR are analysed by PAGE, a multi-banded profile is produced (genetic signature) that reflects the variations present in the DNA sequence under analysis (Pena *et al.*, 1994; Barreto *et al.*, 1996).

The LSSP-PCR technique is capable of detecting single or multiple variations in fragments of target DNA and has already been applied to the study of genetic diseases (Pena *et al.*, 1994), the analysis of polymorphisms in human mitochondrial DNA (Barreto *et al.*, 1996), the genetic characterization of subtypes of human papillomavirus (Villa *et al.*, 1995) and strains of *Entamoeba histolytica* (Gomes *et al.*, 1997) and the molecular typing of strains of *Trypanosoma cruzi* maintained in culture (Vago *et al.*, 1996) or present in tissues of infected animals (Vago *et al.*, 1996; Andrade *et al.*, 1999) and patients with Chagas disease (Vago *et al.*, 2000).

LSSP-PCR has been also used to analyse reference strains of *L. interrogans* (Bao *et al.*, 1997). Similar patterns were obtained in eight serovars analysed, but seven other serovars displayed different LSSP-PCR profiles (Bao *et al.*, 1997).

It should be noted that Gravekamp *et al.* (1993) described the G1/G2-based amplification of DNA fragments of the correct size from six pathogenic species, including *L. meyeri*. This species consists of a pathogenic strain, Iowa City Frog^T (ICF^T), from which a PCR product of the correct size was generated, and a non-pathogenic strain, Veltrat Semarang 173, with the generation of an anomalous PCR product. No G1/G2 amplification was observed from the DNA of strains belonging to the pathogenic species *L. kirschneri* and the non-pathogenic species *L. biflexa*. We found, however, that application of PCR with relatively large quantities of DNA from strains of the three species *L. kirschneri*, *L. biflexa* and *L. meyeri* resulted in the amplification of a product of the correct size in six of these strains, which were used as templates in the LSSP-PCR.

All sequenced fragments originating from different serovars were identical in size (285 bp), indicating that the apparent differences in the electrophoretic mobility of these fragments that had been observed previously (Oliveira *et al.*, 1995) were not related to differences in their size, but possibly to changes in the structure of the DNA due to nucleotide substitutions. These alterations could be generated either by a few nucleotide substitutions, as observed between serovars from the same species, or by many nucleotide substitutions, as observed between DNA fragments of serovars from different genomic species.

Genetic signatures obtained by LSSP-PCR analysis of products pre-amplified by PCR, using either primer G1 or primer G2, consisted of fragments of approximately 100 to 700 bp, with some products consequently larger than the 285 bp used as template. This results from a characteristic of the LSSP-PCR, where it is assumed that the initial products generated in the reaction can act as primers in the subsequent cycles of amplification (Pena *et al.*, 1994; Barreto *et al.*, 1996).

Different genetic signatures were obtained both with the primer G1 as well as with primer G2 in the analysis of serovars obtained from different genomic species of *Leptospira*. However, when the LSSP-PCR profiles generated from serovars belonging to the same genomic species were compared, a clear similarity was apparent, confirming the conservation of the sequence of nucleotides of fragments flanked by the primers G1 and G2.

In agreement with the observations described above, very similar signatures were obtained with primers G1 and G2 for all serovars representing the species *L. interrogans*. Although the sequence data obtained from the 285 bp fragments of three serovars (*icterohaemorrhagiae*, *australis* and *hardjo*) of *L. interrogans* indicated the presence of three nucleotide alterations in these fragments, identical LSSP-PCR profiles were obtained for the three serovars with primer G1 as well as with primer G2. One of the possible explanations for this is the location of these mutations in regions distant from the annealing sites of the primers within the fragment analysed.

A large number of serovars of *Leptospira* have already been described and classified. However, our study was restricted to a small number of serovars representative of the different genomic species, except for the serovars of *L. interrogans*. In this study, the genetic signatures obtained by LSSP-PCR analysis of these serovars presented a satisfactory degree of homogeneity and were sufficient for the characterization and assignment of these serovars to their corresponding genomic species. The characterization of an infective serovar presents an important epidemiological tool for the identification of potential sources of exposure during an outbreak of disease. It will be important to analyse a larger number of serovars representative of diverse genomic species of the genus *Leptospira*.

The LSSP-PCR technique utilized here permits the assignment of the serovars studied into genomic species according to a new proposal of taxonomical classification of the genus *Leptospira*, which is based on intra-specific studies of homogeneity and determination of the G+C contents of genomic DNA (Yasuda *et al.*, 1987). The similarity and consistency of genomic signatures obtained in this study for serovars belonging to the genomic species *L. interrogans* are in total agreement with the results obtained by Yasuda *et al.* (1987), who found 96–99 % homogeneity of DNA for representatives of this genomic species.

LSSP-PCR profiles obtained for the serovars *ndahambukuge* and *grippotyphosa*, both belonging to the genomic species *L. kirschneri*, showed marked differences, which could be due to the presence of little identity in the sequence of the 285 bp genomic fragments used as templates. Our results are in agreement with other studies (Yasuda *et al.*, 1987), which first classified the serovar *grippotyphosa* within the genomic species *L. interrogans*, by demonstrating the existence of 100 % homogeneity at the level of DNA between the serovars *grippotyphosa* and *copenhageni* (*L. interrogans*). In our study, high similarity was observed between the genetic signatures obtained for the serovar *grippotyphosa* (*L. kirschneri*) and for

all serovars of the genomic species *L. interrogans*, including strains M20 and Wijnberg belonging to the serovar *copenhageni*.

Among five non-pathogenic serovars submitted to analysis of homogeneity of DNA (Yasuda *et al.*, 1987), four were analysed in the present study. The serovars *andamana* and *patoc*, belonging to the genomic species *L. biflexa*, presented very similar LSSP-PCR profiles but were distinct from those obtained for the serovars *semaranga* and *ranarum*, which were previously classified within the species *L. biflexa*, but which have been recently reclassified within the genomic species *L. meyeri*, in accordance with differences observed in the G+C content of the DNA. However, according to our results, the serovars *semaranga* and *ranarum* should be classified as belonging to the genomic species *L. biflexa*.

The reproducibility and stability of genetic signatures produced by the LSSP-PCR technique were verified in experiments undertaken on separate days and by using different concentrations of template DNA. Identical signatures were obtained for serovars *canicola*, *australis* and *naam*, all of which belong to *L. interrogans*, which would have permitted the classification of those serovars within the species *L. interrogans*, through the comparison of the profiles with those obtained from other representatives of the group.

Due to their speed and sensitivity, several molecular techniques including the use of serovar-specific probes in DNA hybridization experiments (Yasuda *et al.*, 1987; Terpstra *et al.*, 1986; Zuener & Bolin, 1990), RFLP analysis (Marshall *et al.*, 1984; Ellis *et al.*, 1988) and ribotyping of 16S rRNA (Merien *et al.*, 1992; Ralph *et al.*, 1993; Perolat *et al.*, 1994) have been used in phylogenetic studies and in the identification of strains and serovars of *Leptospira*. Although they are very useful for the genetic identification of serovars of *Leptospira*, many of these techniques present limitations such as being restricted to specific radioactively labelled probes, in addition to the use of complex technical procedures.

Techniques based on the PCR include LS-PCR (low-stringency PCR; Caballero *et al.*, 1994a, b; Brown & Levett, 1997), AP-PCR (arbitrarily primed PCR; Ralph *et al.*, 1993; Perolat *et al.*, 1994) and RAPD (random amplified polymorphic DNA fingerprinting; Corney *et al.*, 1993; Gerritsen *et al.*, 1995). These techniques offer far more precise identification than the LSSP-PCR technique described here. It should be emphasized, however, that AP-PCR, RAPD and LS-PCR can only be applied once the infecting organism has been isolated and cultured and significant quantities of purified DNA have been obtained. In this respect, the value of LSSP-PCR resides in its immediate possibility of application once the specific PCR amplification of a fragment with diagnostic primers G1/G2 has been achieved, even from biological samples.

Furthermore, for those species where the G1/G2 fragment exhibits extensive sequence variability and results in significantly altered LSSP-PCR profiles, this technique also permits serovar identification and thus a rapid means of assessing

potential sources of exposure during outbreaks without the need for DNA probes, restriction analysis or DNA sequencing.

In this work, we have demonstrated the application of LSSP-PCR in the characterization of diverse serovars of the genus *Leptospira* through the analysis of polymorphisms present in a genomic fragment of 285 bp. Due to the simplicity of its execution, its speed and its capacity to produce highly reproducible and informative genetic signatures, we recommend the use of LSSP-PCR for the identification of *Leptospira* and as a valuable tool for the molecular epidemiological study of leptospirosis.

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