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Short communication

Evaluation of LSSP-PCR for identification of *Leptospira* spp. in urine samples of cattle with clinical suspicion of leptospirosis

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Abstract

We evaluated the use of low-stringency single specific primer PCR (LSSP-PCR) for genetically typing *Leptospira* directly from urine samples of cattle with clinical suspicion of leptospirosis. Urine samples obtained from 40 cattle with clinical suspicion of leptospirosis were amplified by specific PCR using the following primers: Internal 1/Internal 2 and G1/G2. The internal primers were designed from the gene sequence of the outer membrane lipoprotein Lip32 from *Leptospira kirschneri*, strain RM52. The PCR products were amplified with these two pairs of primers, which had approximately 497 and 285 bp, respectively, and were subsequently used as a template for LSSP-PCR analysis. The genetic signatures from the leptospires which were present in the urine samples allowed us to make a preliminary identification of the leptospires by comparing the LSSP-PCR profiles obtained directly from urine samples with those from reference leptospires. The LSSP-PCR profiles obtained with the Internal 1 primer or with the G1 primer allowed the grouping of the leptospires into serogroups. LSSP-PCR was found to be a useful and sensitive approach capable of identifying leptospires directly from biological samples without the need for prior bacterial isolation. In conclusion, the LSSP-PCR technique may still be helpful in discriminating serogroups of *Leptospira* from different animal reservoirs, since the early identification of carrier animals and information on the shedding state are crucial to prevent the spread of leptospiral infection to other animals and humans.

Keywords: Bovine; Leptospirosis; LSSP-PCR

1. Introduction

Leptospirosis is a worldwide zoonotic infection. In breeding cows a consequence of leptospirosis is fetal infection with resulting stillbirths, abortions, infertility, early embryonic death, with high economic losses to farmers (Ellis, 1994). The microscopic agglutination test (MAT) is the standard reference test for diagnosis of leptospirosis. This test is laborious and time-consuming, and the interpretation of the results is subjective under dark-field microscopy (Ellis et al., 1991). Definitive diagnosis of leptospirosis is usually provided by culture of the infecting organism. Bacteriological methods for the isolation and identification of

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pathogenic *Leptospira* serovars, however, are slow, labor intensive and are not used for routine diagnosis (Ellis, 1986). For the diagnosis of bovine leptospirosis some investigators have reported the use of molecular approaches for genetic detection and identification of *Leptospira* spp. PCR assays using specific primers to detect *Leptospira interrogans* serovar Hardjo in urine of cattle experimentally infected with Hardjobovis were described by Van Eys et al. (1989) and Gerritsen et al. (1991). Nucleic acid hybridization was used by Lefebvre (1987), Zuerner and Bolin (1988), Bolin et al. (1989) and Wagenaar et al. (2000) for detection of the serovar Hardjobovis. PCR was also used to detect leptospires in bovine semen from animals experimentally infected with *Leptospira* (Heinemann et al., 1999). The LSSP-PCR technique was used to identify serovars pertaining to distinct genomic species of reference *Leptospira* (Oliveira et al., 2003) and leptospires obtained directly from sera of patients clinically suspected of having leptospirosis (Ooteman et al., 2004). The precise identification and classification of leptospires is important for epidemiological and public health surveillance.

In this paper we report the evaluation of LSSP-PCR with the Internal 1 and G1 primers for the identification of leptospires in bovine urine and isolates cultured from urine samples of cattle clinically suspected of having leptospirosis.

2. Materials and methods

2.1. Leptospira serovars

Leptospira serovars were obtained from the reference laboratory of the Royal Tropical Institute of Amsterdam, Netherlands (Table 1) and cultivated in semi-solid and liquid EMJH media (Ellinghausen and Mccullogh, 1965).

2.2. Urine samples from cattle

Urine samples were obtained from cattle suspected of having leptospirosis ($n = 40$) at a dairy farm in the State of Minas Gerais, Brazil. Clinical suspicion was based on finding reproductive problems: repeated failure after artificial insemination or natural breeding, spontaneous abortion, birth of weak calves or mummified fetuses and mastitis. These animals had not been vaccinated against leptospirosis. Urine was collected after washing the vulva of each cow with clean water immediately prior to urine collection. Each urine sample was divided in two portions: (i) an aliquot of 0.5 ml which was immediately inoculated into semi-solid EMJH medium containing 50 μl of 5′fluorouracil (100 μg/ml), 5 μl of neomycin sulphate (300 μg/ml), incubated at 28 °C for up to 6 months and examined periodically to detect leptospiral growth; (ii) the remaining urine sample was transferred into 50 ml disposable plastic centrifuge tubes containing formaldehyde (final concentration, 0.5%) and 4 ml of EDTA (0.5 M, pH 8.0) and kept at −20 °C until DNA extraction. Control urine samples were collected from healthy cattle ($n = 4$) at the Escola de Veterinária da Universidade Federal de Minas Gerais. Antibodies against *Leptospira* spp. were not detected in serum from these animals by MAT (Faine, 1982) or IgG ELISA with recombinant LipL32 antigen (Bomfim et al., 2005). The animals had not been vaccinated against leptospirosis and showed no clinical signs of the disease. All animals were treated according to the "Guide for the Care and Use of Laboratory Animals" of the National Research Council (1996).

2.3. DNA isolation

Genomic DNAs were extracted from 100 μl of pure culture of reference leptospires, 100 μl of each isolate of leptospires cultured from bovine urine ($n = 10$) and the 30 ml of the each bovine urine samples ($n = 40$). DNA extraction was performed by the method described by Boom et al. (1990) with a commercially available DNA extraction kit (BIOMÉRIEUX).

2.4. Specific PCR from all samples tested

PCR reactions were performed with Internal 1 (5′-GAC GGT TTA GTC GAT GGA AAC-3′) and Internal 2 (5′-GGG AAA AGC AGA CCA ACA GA-3′) primers derived from sequence of the LipL32 gene (Haake et al., 2000) that were designed for this work with Oligo 6.0 software (Molecular Biology Insights), and with G1 (5′-CTG AAT CGC TGT ATA AAA GT-3′) and G2 (5′-GGA AAA CAA ATG GTC GGA AG-3′) primers, traditionally known as diagnostic of leptospirosis and described by Gravekamp et al. (1993).

Table 1
Reference *Leptospira* used in this study and the size of their low-stringency single specific primer-PCR (LSSP-PCR) products in pairs of base (bp)

Species/serovars	Strain	Main fragments with Internal 1 primer in (bp[a])	Main fragments with G1 primers in (bp[a])
L. interrogans			
Australis	Ballico	320, 508, 512	180, 190, 220, 280, 420
Australis	Jez Bratislava	170, 190, 480, 512	190, 250, 280
Bataviae	Van Tienen	170, 190, 330, 512	180, 240, 280, 310, 340
Autumnalis	Akiyami A	170, 512	180, 270, 310
Canicola	Hond Utrecht IV	170, 180, 220, 370, 508, 512	180, 240, 270, 310
Icterohaemorrhagiae	M20	170, 220, 290, 320, 440, 512	180, 240, 280, 310
Icterohaemorrhagiae	RGA	170, 180, 220, 270, 330, 380, 430, 512	180, 280
Hebdomadis	Hebdomadis	180, 220, 290, 340, 390, 480, 512	180, 240, 270, 340, 440
Pomona	Pomona	170, 240, 508, 560	180, 240, 270
Djasiman	Djasiman	180, 210, 450, 560	180, 240, 280
Pyrogenes	Salinem	170, 210, 380, 480, 512	180, 240, 270,
Sejroe	3705	170, 508, 512	180, 240, 270, 310
Sejroe	Hardjoprajtino	170, 220, 270, 330, 380, 512	180, 240, 270, 310
L. borgpetersenii			
Ballum	Mus 127	170, 210, 540	180, 240, 280, 400, 420
Ballum	Castellon 3	180, 220, 340, 540	190, 225, 270
Javanica	Veldrat Batavia 46	180, 350, 450, 560	190, 400, 420
Tarassovi	Perepelitsin	220, 450, 620	180, 190, 270, 310
Hardjobovis	Lely 607	180, 190, 220, 270, 470, 512, 520	180, 225, 260, 320, 512
Sejroe	M 84	180, 220, 540	180, 260
L. kirschneri			
Grippotyphosa	Moskva V	170, 180, 512, 520	180, 270, 420, 440
L. santarosai			
Shermani	1342K	180, 220, 320, 330, 380, 480, 520	185, 210, 420

[a]Relative size in base pairs (bp) of main fragments of LSSP-PCR profiles obtained from leptospires using Internal 1 and G1 primers, respectively, run in 6% polyacrylamide silver-stained gel and analyzed with the LabImage—1D gel analysis software, Version 2.7.2. Available in: www.labimage.net.

Amplification of specific PCR products was carried out in a volume of 10 μl containing 5–50 ng of genomic DNA, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide (dNPTs), 0.4 IU Taq DNA polymerase (INVITROGEN-BRL), 1 pmol of each primer in 10 mM Tris–HCl [pH 8.0] and 50 mM KCl, under a layer of 20 μl of 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, 57 °C for 1 min and 72 °C for 1 min. The last cycle consisted of an extension step at 72 °C for 3 min. The same conditions were used for the two different pairs of primers, except for the annealing temperature of the G1 and G2 primers that was 51 °C. The PCR products were run on ethidium bromide-stained gel and the bands corresponding to the specific fragment generated by specific primers (Internal 1/Internal 2 or G1/G2) were excised from the gel with a sterile blade and diluted 1:10 in bi-distilled sterile water. After heating at 95 °C to melt the agarose, 2 μl of eluate was used as the template for the LSSP-PCR assays.

2.5. LSSP-PCR assays

LSSP-PCR was also carried out in a 10 μl volume containing 2 μl of DNA template, 1.5 mM $MgCl_2$, 200 μM of the four deoxynucleotide triphosphates, 1.6 IU Taq DNA polymerase (INVITROGEN-BRL), 48 pmol of Internal 1 or G1 primer in 10 mM Tris–HCl [pH 8.0] and 50 mM KCl, layered with 20 μl of mineral oil. After a denaturation step at 94 °C for 6 min the LSSP-PCR program consisted of 40 cycles of denaturation at 94 °C for 1 min, annealing at 30 °C for 1 min and extension at 72 °C for 1 min. The same

conditions were used for amplification with Internal 2 primer. Five microliters of LSSP-PCR products were analyzed by electrophoresis on 6% (w/v) polyacrylamide gels followed by silver staining (Sanguinetti et al., 1994). After staining, the gels were carefully analyzed in order to determine the main fragments of the LSSP-PCR profiles in comparison with the molecular size of the fragments of a marker 1 kb DNA Ladder (INVITROGEN-BRL). The similarity among the LSSP-PCR profiles of reference leptospires and those obtained with the DNA of the urine samples was analyzed with the LabImage—1D gel analysis software, Version 2.7.2 (Copyright 1999–2004, Kapelan GmbH, Halle (Saale), Germany). Available in: www.labimage.net.

2.5.1. Reproducibility of LSSP-PCR

In order to evaluate the reproducibility and stability of genetic signatures produced by LSSP-PCR with Internal 1 primer, duplicates of the experiments were performed on different days using the same specific PCR products as template. Identical LSSP-PCR patterns were obtained for each distinct sample used regardless of the day the LSSP-PCR reaction was performed (data not shown).

2.6. Dendrogram

LSSP-PCR profiles were scored based on the presence (1) or absence (2) of each polymorphic DNA fragment in all the samples tested with the Internal 1 and G1 primers and a binary matrix of LSSP-PCR phenotypes was assembled. Cluster analysis based on similarity using Nei's (1972) original distance was performed by the Unweighted Pair Group Method (UPGMA) with arithmetic averages clustering algorithm (Sneath and Sokal, 1973) and the randomization procedure as implemented in Tools for Population Genetic Analyses (TFPGA) software package (Miller, 1998).

2.7. Purification and sequencing of PCR products

In order to determine nucleotide sequence of the 497 fragments generated with Internal 1 and Internal 2 primers, 4 DNA samples were chosen randomly for sequencing out of 10 isolates of leptospires cultured from bovine urine. In addition, PCR assays were also performed with DNA from *L. borgpetersenii* serovar Hardjobovis, strain Lely 607 and from one bovine urine sample in which leptospires had not been isolated. This sample was from animal number 21. The PCR products were resolved in agarose gels (1.5%) stained with ethidium bromide (0.5 μg/ml). The electrophoretic run was in TBE buffer (0.45 mM tris-borate, 0.001 mM EDTA) at 100 V for 2 h. Specific fragments were excised from the gel with a sterile blade and purified with GFX™ PCR DNA and Gel Band Purification kit (AMERSHAM BIOSCIENCES) and sequenced according to the method originally described by Sanger et al. (1977). Nucleotide sequences determined by the Automated Mega BACE 1000TM (AMERSHAM BIOSCIENCES) sequencing system were edited with MEGA program Version 2.1 (Kumar et al., 2001). Aligned with *Leptospira* LipL32 sequence from the Genbank database was done with the CLUSTALW software package (EMBL-EBI) (http://www.ebi.ac.uk/clustalw/). The sequences were analyzed for homology with database sequences with BLASTn of the (BASIC ALIGNMENT SEARCH TOOL) http://www.ncbi.nlm.nih.gov/BLAST.

3. Results

3.1. PCR assays

A 497 bp fragment was specifically amplified from all serovars of *Leptospira* used in this study (Table 1) by PCR with the Internal 1/Internal 2 primers. Fragments that varied from 290 to 310 bp were also obtained with G1 and G2 primers using the same samples. From the 40 bovine urine samples submitted to the specific PCR with Internal 1/Internal 2 and G1/G2 primers, 21 (52.5%) showed amplification products. Furthermore, of these 21 urine samples specifically amplified were the 10 bovine samples from which leptospires had been isolated. These fragments were further characterized using LSSP-PCR reaction with Internal 1, Internal 2 or G1 primers.

3.2. LSSP-PCR

The Internal 2 primer showed LSSP-PCR profiles that were complex and identical but this did not permit differentiation between serovars (data not shown).

3.2.1. LSSP-PCR with Internal 1 primer

Electrophoretic multiple band profiles resulting from LSSP-PCR for 21 leptospiral reference strains were generated with the use of the Internal 1 primer. Each of the four species revealed a distinct pattern (Fig. 1A). The electrophoretic migration profile of the amplified fragments varied between the different species evaluated. Molecular size, determined by the LabImage software, for serovars of *L. interrogans* was 170–560 bp and for serovars of *L. borgpetersenii* the size was 170–620 bp. For serovars of *L. santarosai* the molecular size was of 180–520 bp and for serovars of *L. kirschneri* was 170–520 bp. Table 1 shows the molecular size of the main fragments that compose the LSSP-PCR profiles for each serovar. Comparison of *L. interrogans* serovar Hardjoprajitno and *L. borgpetersenii* serovar Hardjobovis revealed different patterns (Fig. 1A, lanes 7 and 17). No amplification was observed when DNA from non-pathogenic *L. biflexa* serovar Andamana (strain CH 11) was used as DNA templates (data not shown).

3.2.2. LSSP-PCR with Internal 1 primer and DNA from clinical samples

Fig. 1B shows the LSSP-PCR profiles of DNA obtained from leptospires present in the 21 urine samples of cattle suspected of having leptospirosis and amplified with Internal 1 primer. Fig. 2A shows the

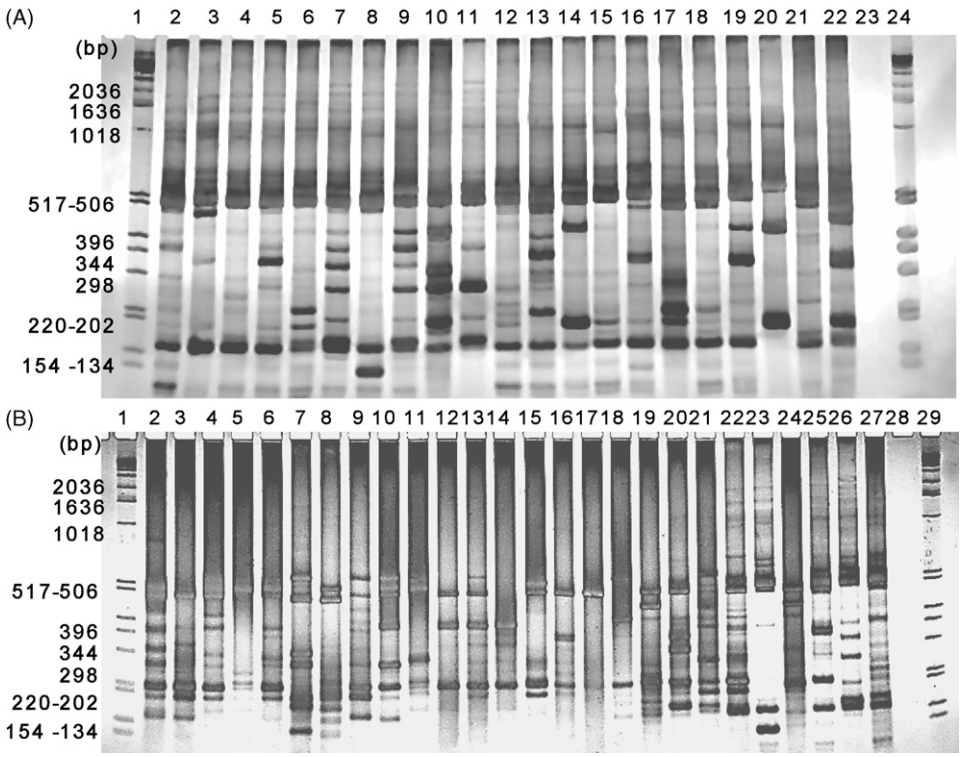

Fig. 1. (A) Silver-stained 6% polyacrylamide gel showing the LSSP-PCR profiles from different species of reference *Leptospira* amplified with Internal 1 primer. Lanes 1 and 24, molecular size marker (1 kb DNA Ladder). Lanes 2–14, *L. interrogans*, serovars Australis, Bratislava, Autumnalis, Bataviae, Canicola, Hardjoprajitno, Wolffi, Icterohaemorrhagiae, Copenhageni, Hebdomadis, Pyrogenes, Pomona and Djasiman. Lanes 15–20, *L. borgptersenii* serovars Ballum, Castellonis, Hardjobovis, Sejroe, Javanica and Tarassovi. Lane 21, *L. kirschneri* serovar Grippotyphosa. Lane 22, *L. santarosai* serovar Shermani. Lane 23, control of the reagents used for the reaction of LSSP-PCR. (B) Silver-stained 6% polyacrylamide gel showing the LSSP-PCR profiles from DNA extracted of urine samples from bovines suspected of having leptospirosis and amplified with Internal 1 primer. Lanes 1 and 29, molecular size marker (1 kb DNA Ladder). Lanes 2–22, samples from animals numbers: 497, 236, 28, 52, 154, 15, 09, 21, 41, 26, 204, 182, 01, 92, 119, 261, 02, 05, 30, 32 and 08, respectively. Lane 23, *L. interrogans* serovar Wolffi. Lane 24, *L. borgpetersenii* serovar Hardjobovis. Lane 25, *L. interrogans* serovar Pomona. Lane 26, *L. kirschneri* serovar Grippotyphosa. Lane 27, *L. interrogans* serovar Hardjo. Lane 28, control urine from a bovine negative for leptospirosis.

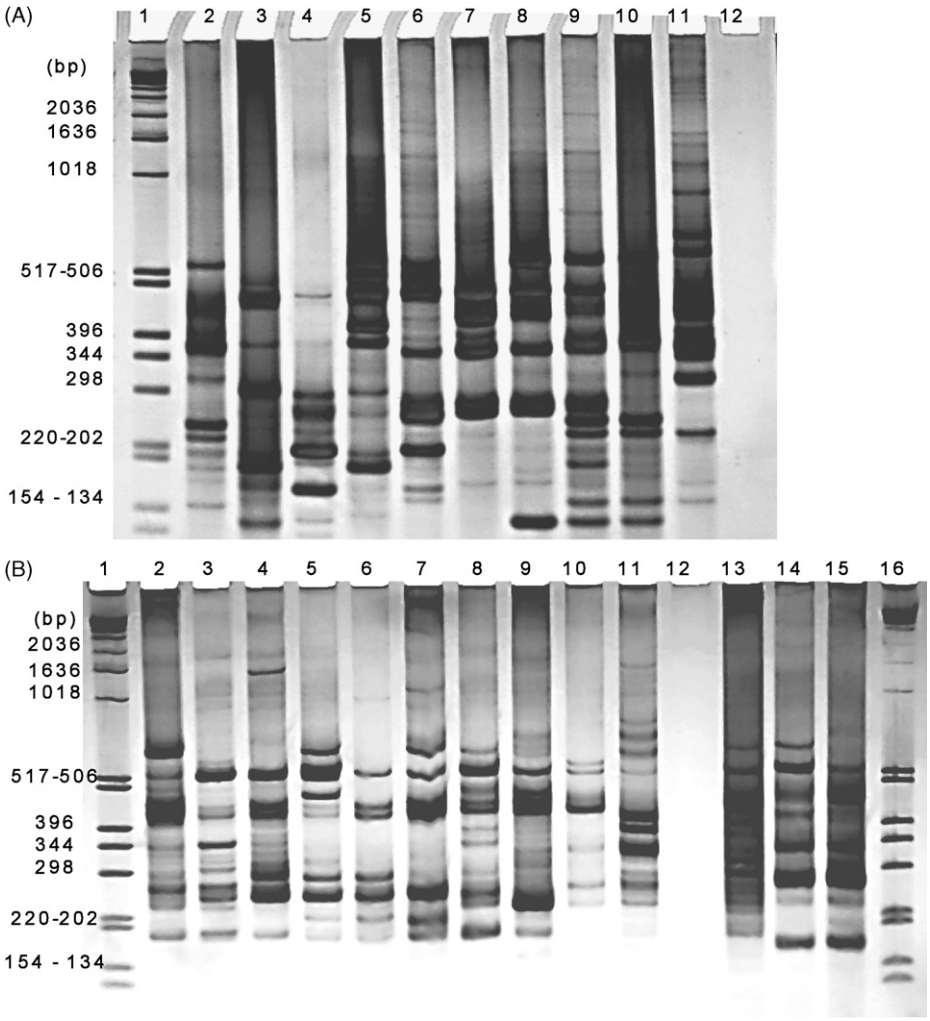

Fig. 2. (A) Silver-stained 6% polyacrylamide gel showing the LSSP-PCR profiles from DNA extracted of the isolates of leptospires cultured from bovine urine in semi-solid EMJH medium and amplified with Internal 1 primer. Lane 1, molecular size marker (1 kb DNA Ladder). Lanes 2–11, leptospires isolated from urine samples of the animals numbers: 09, 497, 28, 52, 154, 182, 15, 204, 236 and 01. Lane 12, control of the reagents used for the reaction of LSSP-PCR. (B) Silver-stained 6% polyacrylamide gel showing the LSSP-PCR profiles from DNA extracted of leptospire isolates cultured from bovine urine in medium EMHJ and amplified with primer G1. Lanes 1 and 16, molecular size marker (1 kb DNA Ladder). Lanes 2–11, profiles presented by samples from animals numbers: 09, 497, 28, 52, 154, 182, 15, 204, 236 and 01. Lane 12, control of the reagents used for the reaction of LSSP-PCR. Lane 13, *L. kirschneri* serovar Grippotyphosa. Lane 14, *L. borgpetersenii* serovar Hardjobovi*s.* Lane 15, *L. interrogans* serovar Wolffi.

DNA profiles of the isolates of leptospires cultured from bovine urine in semi-solid EMJH medium. The main fragments analyzed by LabImage software were composed of fragments ranging from approximately 180 to 520 bp for DNA from urine bovine and the fragments ranging from approximately 180 to 540 bp with DNA for leptospire isolates (Table 2). The major bands of the LSSP-PCR patterns obtained from reference *Leptospira* serovars (Fig. 1A and Table 1) were compared with those from the DNA from bovine urine (Figs. 1B and 2A, Table 2). The LSSP-PCR profiles of these samples were clustered in two groups (profiles 1 and 2) according to the largest number of fragments coinciding with the fragments from the reference *Leptospira* profile. Profile 1 represents the group of samples that presented the highest number of

Table 2
Main fragments of low-stringency single primer-PCR (LSSP-PCR) profiles obtained with DNA of bovine urine samples and the isolates of leptospires cultured from bovine urine and amplified with Internal 1 primer and G1 primer in pairs of base (bp)

Number of the animals	Internal 1 primer	G1 primer	Reference *Leptospira* serovars with Internal 1 and G1
Profile 1			
28	180, 230, 280, 512[a]	180, 270, 420, 440[a]	*L. kirschneri* serovar Grippotyphosa with Internal 1 (170, 180, 512, 520) G1 primer (180, 270, 420, 440)
	180, 230, 280, 512, 520[b]	270, 420, 440, 512[b]	
Profile 2			
01	190, 300, 350, 470[a]	225, 260, 320, 512[a]	*L. borgpetersenii* serovar Hardjobovis with Internal 1 primer (180, 190, 220, 270, 470, 512, 520)
	250, 300, 350, 470, 520[b]	260, 320, 512[b]	
09	180, 190, 470, 512[a]	225, 260, 320, 440, 512[a]	*L. borgpetersenii* serovar Sejroe with Internal 1 primer (180, 220, 540)
	180, 190, 220, 300, 350, 470, 512[b]	180, 225, 260, 440, 512[b]	
15	180, 190, 250, 300, 470, 512[a]	225, 260, 320, 440, 470[a]	*L. borgpetersenii* serovar Hardjobovis with G1 primer (180, 225, 260, 320, 512)
	180, 220, 270, 300, 470[b]	225, 260, 400, 440, 512[b]	
52	220, 512, 520[a]	260, 320, 440, 512, 600[a]	*L. borgpetersenii* serovar Sejroe with G1 primer (180, 260)
	220, 270, 350, 470, 512[b]	240, 260, 440, 512, 600[b]	
154	190, 220, 270, 350, 470, 512[a]	260, 320, 440, 512[a]	
	270, 350, 470[b]	240, 260, 420, 440, 512[b]	
182	220, 360, 470[a]	225, 260, 320, 420, 512[a]	
	270, 360, 450, 470, 512[b]	225, 240, 260, 420, 440, 512[b]	
204	220, 350, 470[a]	225, 260, 320, 420[a]	
	220, 250, 270, 350, 470, 520[b]	420, 440, 512[b]	
236	190, 220, 250, 300, 470[a]	260, 320[a]	
	220, 250, 350, 520[b]	420, 512[b]	
497	190, 220, 250, 300, 350, 470, 512[a]	180, 260, 320, 420, 512[a]	
	300, 350, 520, 540[b]	260, 340, 420, 512[b]	
26	220, 270, 470, 512[a]	180, 200, 225, 260, 320, 440, 512[a]	
92	190, 220, 470[a]	225, 260, 320[a]	
119	220, 350, 470[a]	225, 320, 440[a]	
02	220, 380, 470[a]	225, 260, 440, 512[a]	
05	180, 220, 470, 512[a]	225, 260, 512[a]	
08	190, 220, 270, 350, 470, 512[a]	225, 260, 320[a]	
30	180, 220, 270, 300, 512[a]	225, 260, 320[a]	
32	180, 220, 270, 350, 470, 520[a]	225, 260, 320[a]	
261	220, 470[a]	260, 320, 420[a]	
41	220, 250, 350, 470, 512[a]	225, 260, 320, 420[a]	
21	180, 190, 220, 250, 470, 520[a]	260, 280, 370[a]	

[a] DNA urine samples.
[b] DNA isolates of leptospires.

fragments in agreement with serovar Grippotyphosa of *L. kirschneri* and profile 2 represents the group of the samples that had shown the greatest number of fragments coincident with serovars pertaining to serogroup Sejroe, especially with serovar Harjobovis of *L. borgpetersenii* (Table 2).

3.3. LSSP-PCR with G1 primer and DNA Leptospira reference and clinical samples

Informative genetic signatures exhibiting multiple bands were obtained for all reference serovars analyzed (their main fragments are listed in Table 2). LSSP-PCR patterns that were similar to each other were observed mainly among serovars of *L. interrogans* (Fig. 3A, lanes 2–14) and *L. borgpetersenii* (lanes 15–17) serovars Ballum, Castellonis and Hardjobovis.

LSSP-PCR profiles obtained with DNA extracted from bovine urine samples and amplified with primer G1 are shown in Fig. 3B. In addition, Fig. 2B shows the profiles from DNA of the isolates of leptospires cultured from bovine urine. Table 2 shows the molecular size of the fragments analyzed by the LabImage software, those DNA fragments of greater

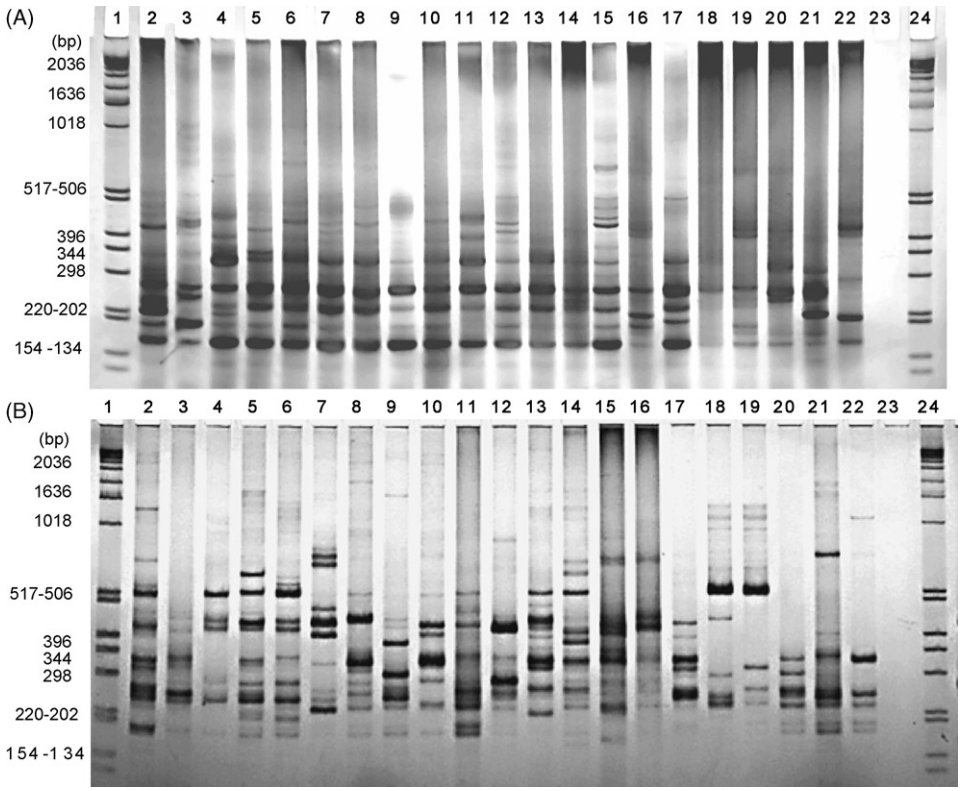

Fig. 3. (A) Silver-stained 6% polyacrylamide gel showing the LSSP-PCR profiles of DNA extracted from different species of reference *Leptospira* and amplified with primer G1. Lanes 1 and 24, molecular size marker (1 kb DNA Ladder). Lanes 2–14, *L. interrogans*, serovars Australis, Bratislava, Autumnalis, Bataviae, Canicola, Hardjoprajitno, Wolffi, Icterohaemorrhagiae, Copenhageni, Hebdomadis, Pyrogenes, Pomona and Djasiman. Lanes 15–20, *L. borgptersenii* serovars Ballum, Castellonis, Hardjobovis, Sejroe, Javanica and Tarassovi. Lane 21, *L. kirschneri*, serovar Grippotyphosa. Lane 22, *L. santarosai* serovar Shermani. Lane 23, control of the reagents used for the reaction of LSSP-PCR. (B) Silver-stained 6% polyacrylamide gel showing the LSSP-PCR profiles of DNA extracted from urine samples of bovines suspected of having leptospirosis and amplified with primer G1. Lanes 1 and 24, molecular size marker (1 kb DNA Ladder); lanes 2–23, profiles presented by samples from animals numbers 497, 236, 28, 52, 154, 15, 09, 21, 41, 26, 204, 182, 01, 92, 119, 261, 02, 05, 30, 32 and 08, respectively. Lane 23, control urine from a bovine negative for leptospirosis = samples of the Minas Gerais State.

intensity were considered as the major bands. The gene signatures obtained through the LSSP-PCR with DNA urine samples were composed of multiple fragments varying from 180 to 732 bp and for reference serovars varying from 180 to 512 bp. The similarity between LSSP-PCR profiles was verified by comparison of the size (in base pairs) and the number of the main DNA constituent fragments in each LSSP-PCR pattern obtained from the biological samples with those shown by reference leptospira serovars (Fig. 3A and Table 1). We were able to group the LSSP-PCR profiles into two distinct groups (profiles 1 and 2) according to the largest number of fragments coinciding with fragments of the reference leptospira profiles. The results of G1 primer for these samples were similar to those obtained with Internal 1 primer.

4. Dendrogram

Fig. 4 shows a dendrogram obtained by the UPGMA clustering algorithm, that grouped the results of LSSP-PCR according to phenotypical similarity among profiles generated by Internal 1 and G1 primers for reference serovars and those profiles produced by 21 DNA urine samples from cattle suspected of being infected with leptospirosis, as previously shown in Fig. 1A and Tables 1 and 2, respectively. This

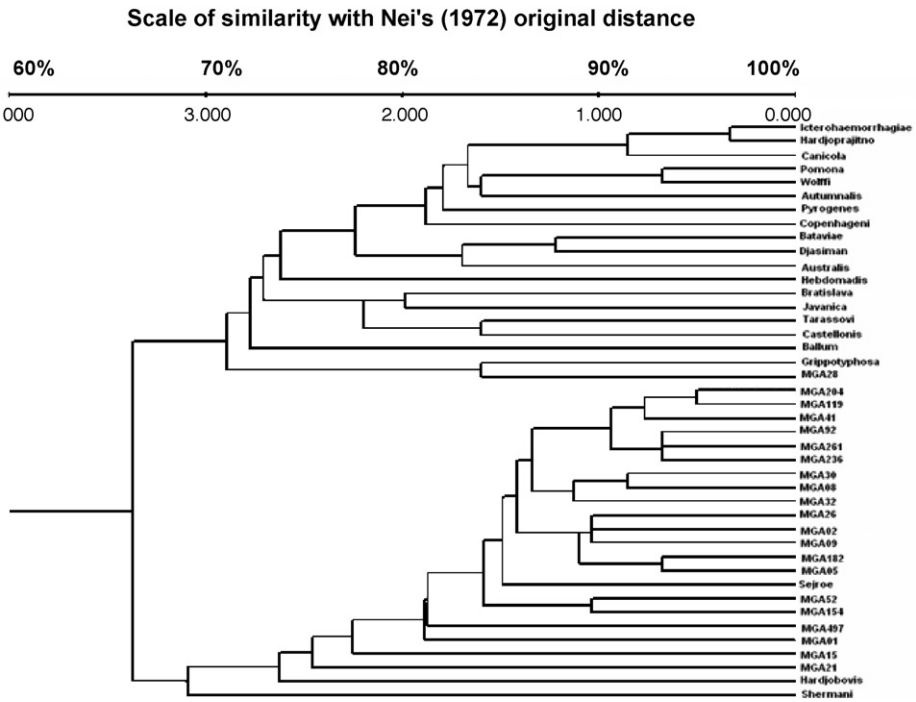

Fig. 4. Dendrogram of the UPGMA cluster analysis resulting from the comparison of the electrophoretic patterns obtained by LSSP-PCR with G1 and Internal 1 primers from 21 reference serovars of four genomic species *Leptospira* and the profiles of LSSP-PCR with G1 and Internal 1 primers from 21 DNA bovine urine samples from animals suspected of having leptospirosis. Abbreviations: MGA = samples of the Minas Gerais State.

dendrogram was generated based on the data matrix (presence or absence of specific fragments) with each primer. The dendrogram constructed with the results obtained with two primers in combination using the similarity of Nei (1972) original distance allow the clustering the samples into two groups according to the similarity between them. Group 1 contains only the sample (MGA 28) corresponding to serovar Grippotyphosa of *L. kirschneri*. Group 2 contains 20 samples that presented similar profiles to serovars pertaining to serogroup Sejroe, especially with serovar Hardjobovis of *L. borgpeterseni*.

5. Sequencing

All fragments sequenced with Internal 1 and Internal 2 primers and originating from one urine sample of a cow suspected of having leptospirosis, plus four samples of leptospiral isolates cultured from bovine urine and the *L. borgpetersenii* serovar Hardjobovis were of the same size (497 bp). The sequences of the PCR products from these samples were deposited on Genbank (accession numbers: DQ286415, DQ286416, DQ286417, DQ286418, DQ320625 and DQ343231). These sequences generated with Internal 1 and Internal 2 primers were compared with 34 sequences of the LipL32 from reference serovars in Genbank belonging to six pathogenic species of *Leptospira* (*L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. noguchii* and *L. wellii*). The results of the similarity search obtained by BLASTn alignment indicated that two isolates of leptospires cultured from bovine urine in EMHJ medium, plus one leptospire sample from bovine urine, had the highest identity with different serovars of *L. borgpetersenii*. Another sample showed similarity with different serovars of *L. interrogans* and only one sample showed similarity with serovars of *L. kirschneri.* The sequence identities shared with serovars of *L. borgpetersenii* consistently varied from 96.0 to 99.0%, while that for serovars of *L. kirschneri*

varied from 97.0 to 98.0%. Identities with sequences from serovars of other species varied from 96 to 97%.

6. Discussion

In this work we have demonstrated the application of the LSSP-PCR technique for the characterization of leptospires directly from bovine urine samples through the analysis of polymorphisms present in a genomic fragment of *Leptospira.* Primers specially designed from the sequence of the lipoprotein LipL32, were used here for the first time in the LSSP-PCR to identify reference *Leptospira*, isolates of leptospires cultured from bovine urine and leptospires present in urine samples of cattle suspected of having leptospirosis. One of the main advantages of using LSSP-PCR for genetically typing *Leptospira* from biological samples is the possibility to detect leptospires first by means of a PCR reaction and subsequently identify them through the comparison of their genetic signatures with known LSSP-PCR profiles obtained from reference serovars.

LSSP-PCR with Internal 1 primer showed a sensitivity of 100% when amplifying eluates of the fragments generated for specific PCR with Internal 1 and Internal 2 primers. By comparison among LSSP-PCR genetic signatures obtained with Internal 1 primer using DNA from reference *Leptospira* serovars and those from clinical samples it is suggested that two species *L. borgpetersenii* and Grippotyphosa of *L. kirschneri* could have infected the study herd. The same results were found using LSSP-PCR with G1 primer and DNA from these samples. In comparison to the signatures obtained with G1 from reference serovars (Table 1), the LSSP-PCR profiles were very similar and presented the highest number of coincident fragments specially for serovars Hardjobovis and Sejroe of serogroup Sejroe (Figs. 2B and 3B, Table 2).

We obtained good reproducibility in the LSSP-PCR profiles even when primer G1 and the LSSP-PCR were used to type leptospires directly from bovine urine samples and from isolates of leptospires cultured from bovine urine with distinct DNA templates that had been amplified on different days (data not shown).

The analysis of the alignment of LipL32 sequence of the DNAs from clinical isolates and bovine urine in relation to consensus sequence indicated the presence of some nucleotide substitutions. However, these polymorphisms detected were single-nucleotide and all were silent. Identical results have been obtained by Haake et al. (2000) when comparing different sequences of LipL32. For the authors these substitutions suggest that there is evolutionary pressure to maintain the primary sequence of this protein. In addition, sequence analysis indicated that LSSP-PCR was capable of grouping infecting serogroups in all sequenced samples with internal primers designed from the LipL32 amino acid sequence. Our results of comparative sequence analysis for the LipL32 amino acid sequence are in agreement with Haake and Matsunaga (2005) who found an average of 99.1% for the same species of pathogenic *Leptospira.*

In this work we tested the use of LSSP-PCR for genetically typing leptospires directly from urine samples of cattle with clinical suspicion of leptospirosis and analyzed isolates of leptospires cultured from bovine urine in semi-solid EMJH medium. This has not been previously described by other authors.

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References

Bomfim, M.R.Q., Ko, I.A., Koury, M.C., 2005. Evaluation of the recombinant LipL32 in enzyme-linked immunosorbent assay for the serodiagnosis of bovine leptospirosis. Vet. Microbiol. 109, 89–94.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M.E., Van Noordaa, J., 1990. Rapid and simple method for purification of nucleic acids. Infect. Immun. 28, 495–503.

Bolin, C.A., Zuerner, R.L., Trueba, G., 1989. Comparison of three techniques to detect *Leptospira interrogans* serovar *hardjo* tipo *hardjo-bovis* in bovine urine. Am. J. Vet. Res. 50, 1001–1003.

Ellinghausen Jr., H.C., Mccullogh, W.G., 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: a serum-free medium employing oleic albumin complex. Am. J. Vet. Res. 26, 39–44.

Ellis, W.A., 1986. The diagnosis of leptospirosis in farm animals. In: Ellis, W.A., Little, T.W.A. (Eds.), The Present State of Leptospirosis Diagnosis and Control. Martinus Nijhoff, Dordrecht, The Netherlands, pp. 13–31.

Ellis, W.A., Montgnomery, J.M., Thierman, A.B., 1991. Restriction endonuclease analysis as a taxonomic tool in the study of pig isolates belonging to the *australis* serogroup of *Leptospira interrogans*. J. Clin. Microbiol. 29, 957–961.

Ellis, W.A., 1994. Leptospirosis as a cause of reproductive failure. Vet. Clin. North Am.: Food Anim. Pract. 10, 463–478.

Faine, S., 1982. Guideline for Leptospirosis Control, vol. 67. WHO Offset publications, Geneva, p. 172.

Gerritsen, M.J., Olyhoek, T., Smits, M.A., Bokhout, B.A., 1991. Sample preparation method for polymerase chain reaction-based semiquantative detection of *L. interrogans* serovar *hardjo* subtype *hardjobovis* in bovine urine. J. Clin. Microbiol. 29, 2805–2808.

Gravekamp, C., Van de Kemp, H., Franzen, M., Carrington, D., Van Eys, G.J.J.M., Everard, C.O.R., Hartskeerl, R.A., Terpstra, W.J., 1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J. Gen. Microbiol. 139, 1691–1700.

Guide for the Care, 1996. Use of Laboratory Animals. National Research Council, Washington.

Haake, D.A., Chao, G., Zuerner, R.L., Barnett, J.K., Barnett, D., Mazel, M., Matsunaga, J., Levett, P.N., Bolin, C.A., 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect. Immun. 68, 2276–2285.

Haake, D.A., Matsunaga, J., 2005. Leptospiral membrane proteins—variations on a theme. Indian J. Med. Res. 121, 143–145.

Heinemann, M.B., Garcia, J.F., Nunes, C.M., Morais, Z.M., Gregori, F., Cortez, A., Vasconcellos, S.A., Visitin, J.A., Richtzenhain, L.J., 1999. Dectection of leptospires in bovine semen by polymerase chain reaction. Aust. Vet. J. 77, 32–34.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Arizona State University, Tempe, AZ, USA.

Lefebvre, R.B., 1987. DNA probe for the detection of the *Leptospira interrogans* serovar *hardjo* serotype hardjobovis. J. Clin. Microbiol. 25, 2236–2238.

Miller, M.P., 1998. TFPGA: Tools for Population Genetic Analyses for Windows. Arizona State University, USA.

Nei, M., 1972. Genetic distance between populations. Am. Nat. 106, 283–292.

Oliveira, M.A.A., Caballero, O.L.S.D., Vago, A.R., Hartskeerl, R., Romanha, A.J., Pena, S.D.J., Simpson, A.J.G., Koury, M.C., 2003. Low-stringency single specific primer PCR for identification of *Leptospira*. J. Med. Microbiol. 52, 127–135.

Ooteman, M.C., Vago, A.R., Koury, M.C., 2004. Potential application of low-stringency single specific primer-PCR in the identification of *Leptospira* in the serum of patients with suspected leptospirosis. Can. J. Microbiol. 12, 1073–1079.

Sanguinetti, C.J., Dias Neto, E., Simpson, A.J.G., 1994. Rapid silver staining and recovery of PCR products separated on polyacrilamide gels. Biotechniques. 17, 915–918.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467.

Sneath, P.H.A., Sokal, R.R., 1973. Numerical taxonomy. Freeman, San Francisco, p. 573.

Van Eys, G.J.J.M., Gravekamp, C., Gerritsen, M.J., Quint, W., Cornelissen, M.T.E., 1989. Detection of leptospires in urine by polymerase chain reaction. J. Clin. Microbiol. 27, 2258–2262.

Wagenaar, J.A., Zuerner, R.L., Alt, D., Bolin, C.A., 2000. Comparison of polymerase chain reaction assays with bacteriologic culture, immunofluorescence, and nucleic acid hybridization for detection of *Leptospira* borgpetersenii serovar hardjo in urine of cattle. Am. J. Vet. Res. 61, 316–320.

Zuerner, R.L., Bolin, C.A., 1988. Repetitive sequence element cloned from *Leptospira interrogans* serovar *hardho* type hardjobovis provides a sensitive diagnostic probe for bovine leptospirosis. J. Clin. Microbiol. 26, 2495–2500.