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Low-Stringency PCR with Diagnostically Useful Primers for Identification of *Leptospira* Serovars

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Primers proposed for the diagnosis of the pathogenic spirochete *Leptospira* spp. (C. Gravekamp, H. V. D. Kemp, M. Franzen, D. Carrington, G. J. Schoone, G. J. J. M. Van Eys, C. O. R. Everard, R. A. Hartskeel, and W. J. Terpstra, J. Gen. Microbiol. 139:1691–1700, 1993) have been found to produce complex serovar-specific patterns under low-stringency PCR conditions. Such patterns obtained by low-stringency PCR, which maintain the specific band as an internal control, offer an approach to the standardized identification of *Leptospira* serovars in clinical laboratories.

Spirochetes of the genus *Leptospira* are composed of seven officially recognized pathogenic genomic species and four nonpathogenic genomic species which are divided into more than 250 serovars by serotypic classification on the basis of microagglutination (2, 6). The precise identification and classification of *Leptospira* spp. is important for epidemiological and public health surveillance since different serovars exhibit different host specificities and are responsible for different clinical forms of the disease (2, 8). To explore the genetic basis of serovar variation and to facilitate the identification of serovars, a number of DNA-based methodologies have been elaborated (4, 7–10). The majority of these methodologies are not ideally suited to clinical laboratories concerned with serovar identification because of their complexity and the relatively large amounts of DNA required from this slow-growing organism. The most potentially useful approach for clinical identification so far proposed is that of arbitrarily primed PCR (AP-PCR) because of its simplicity, the fact that only nanogram quantities of *Leptospira* DNA are required, and its sensitivity to genomic variation (10). The method uses randomly selected primers to amplify anonymous regions of the *Leptospira* genome. The amplified products are highly polymorphic and appear to constitute serovar-specific PCR fingerprints. Although within a species a number of bands are shared by different serovars, the fingerprints differ markedly between different genomic species. Potential complications of the technique, however, are that relatively distant serovars have no PCR products in common and that there is no internal control to verify that the DNA being amplified is indeed derived from *Leptospira* spp. These are important considerations, since arbitrarily selected primers may amplify regions of any genome under the low-stringency conditions used for AP-PCR and there is no a priori means of determining whether an unexpected fingerprint derives from an unusual serovar or from DNA that has resulted from a laboratory mix-up. A possible solution to this problem is the use of specific primers, such as those that are used for diagnosis (3), for low-stringency amplification. We have termed the use of specific primers under low-stringency conditions low-stringency PCR (LS-PCR) (1), which differs from AP-PCR in that the choice of primer is no longer arbitrary, although the conditions of the reaction remain the same. When specific primers are used under low-stringency conditions (i.e., for LS-PCR), the result is not only the amplification of the specific DNA sequence defined by the primers (which thus identifies the source of the DNA and acts as a control for contamination) but also a set of a low-stringency products (LSPs), such as those that are produced by AP-PCR. In the study described here, we investigated this approach using a collection of the *Leptospira* serovars commonly isolated in Brazil (11) together with representatives of all commonly occurring serogroups. The primers used were the G1-G2 pair described by Gravekemp and colleagues (3) for the diagnosis *Leptospira* infection. We show that the LS-PCR profiles produced vary between the serovars tested and that the specific amplification product is indeed preserved in all cases.

Isolates of the various *Leptospira* serovars were cultured in the liquid medium of Ellinghausen and McCullough as modified by Johnson and Harris (5) for 7 to 10 days under aerobic conditions in the dark at 28°C. During the exponential growth phase, the organisms were centrifuged at 13,000 × *g* for 30 min at 4°C, and the pellet was washed twice in phosphate-buffered saline (PBS) and stored at −70°C until use. Serovar stocks were maintained on semisolid medium (2). Table 1 lists the *Leptospira* serovars used in the study and their origins. Total DNA was prepared by cell lysis and overnight incubation in sodium dodecyl sulfate proteinase K; this was followed by extraction with phenol-chloroform and ethanol precipitation as described by Tamai et al. (13). The DNA was resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 8.0) and was stored at 4°C. The concentration of DNA was determined by electrophoresis against known standards, and the amounts indicated in the text were used for amplification. LS-PCR was undertaken with the primers previously described by Gravekamp et al. (3) for the specific diagnosis of *Leptospira* spp.: G1, 5′-CTGAATCGCT GTATAAAAGT-3′; G2, 5′-GGAAAACAAATGGTCGGA AG-3′. A reaction volume of 10 μl was used for all amplifications and contained 200 μM (each) the four deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM

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TABLE 1. *Leptospira* serovars used in the study

Serogroup	Serovar	Strain	Genomic species[a]
Andamana[b]	Andamana	CH-11	*L. biflexa*
Australis[b,c]	Australis	Ballico	*L. interrogans*
Autumnalis[b]	Autumnalis	Akiyiami A	*L. interrogans*
Ballum[b]	Castellonis	Castellón 3	*L. borgpetersenii*
Bataviae[b]	Bataviae	Van Tienen	*L. interrogans*
Canicola[c]	Bafani	Bafani	*L. kirshneri*
Canicola[b]	Canicola	Hond Utrecht	*L. interrogans*
Canicola[c]	Schueffneri	Vleermuis	*L. interrogans*
Canicola[c]	Sumneri	Sumner	*L. kirshneri*
Celledoni[b]	Celledoni	Celledoni	*L. weilli*
Cynopteri[b]	Cynopteri	3522 C	*L. kirshneri*
Djasiman[b,c]	Djasiman	Djasiman	*L. interrogans*
Djasiman[b]	Sentot	Sentot	*L. interrogans*
Grippotyphosa[b]	Grippothyphosa	Moskva V	*L. kirshneri*
Hebdomadis[c]	Hebdomadis	Hebdomadis	*L. interrogans*
Icterohaemorrhagiae[c]	Copenhageni	M 20	*L. interrogans*
Icterohaemorrhagiae[c]	Icterohaemorrhagiae	RGA	*L. interrogans*
Icterohaemorrhagiae[b]	Icterohaemorrhagiae	3294	NA[d]
Icterohaemorrhagiae[c]	Naan	Naan	*L. interrogans*
Icterohaemorrhagiae[c]	Ndahambukuge	Ndahambukuge	*L. kirshneri*
Javanica[b]	Javanica	Veldrat Bataviae 46	*L. borgpetersenii*
Panama[c]	Panama	CZ 214	*L. noguchi*
Pomona[c]	Pomona	Pomona	*L. interrogans*
Pyrogenes[b,c]	Pyrogenes	Salinem	*L. interrogans*
Sarmin[b]	Sarmin	Sarmin	*L. weilli*
Sejroe[c]	Hardjo	Hardjoprajitno	*L. interrogans*
Sejroe[b]	Sejroe	M 84	*L. borgpetersenii*
Semaranga[b,c]	Patoc	Patoc I	*L. biflexa*
Shermani[b]	Shermani	LT 821	*L. santarosai*
Tarassovi[b]	Tarassovi	Mitis Johnson	NA

[a] Genomic species according to references 9 and 14.
[b] Strains provided by the Leptospirosis Laboratory, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil.
[c] Strains obtained from the Culture Collection of the Instituto Adolfo Lutz, São Paulo, Brazil.
[d] NA, not available.

Tris-HCl (pH 8.5), together with 6 pmol of each primer. One nanogram of DNA and 0.8 U of *Taq* polymerase (a kind gift from the Center for Biotechnology, Rio Grande do Sul, Brazil) were then added, and the reaction mixture was overlaid with 20 μl of mineral oil. The first cycle of amplification consisted of denaturation at 95°C for 5 min, annealing at 30°C for 2 min, and extension at 72°C for 3 min. The second cycle was identical to the first one, except that the denaturation step was at 92°C for 45 s. The subsequent 33 cycles consisted of denaturation at 92°C for 45 s, primer annealing at 40°C for 1 min, and extension at 72°C for 2 min. An additional 5 min at 72°C was included at the end of the program to ensure complete extension. Amplified samples were subjected to electrophoresis by using 10% polyacrylamide gels and then silver staining (12).

For specific amplification, 1 pmol of primer and 0.4 U of *Taq* polymerase per 10-μl reaction mixture were used. The other components of the reaction mixture were as described above for LS-PCR. The specific amplification cycle consisted of an initial denaturation step at 94°C for 3 min; this was followed by 30 cycles of amplification of 51°C for 2 min, 72°C for 2 min, and 94°C for 30 s, with an extended incubation at 72°C for 5 min in the final cycle.

Specific amplification of all the *Leptospira* serovars listed in Table 1 with the primers G1-G2 was found to result in a major DNA product of approximately 290 bp together with a shadow band of approximately 750 bp, as revealed on silver-stained polyacrylamide gels (Fig. 1A). The group of organisms tested included organisms of serovars that were not originally reported to be amplifiable by G1-G2 (3). This difference may be due to either the slightly lower annealing temperature or, possibly, the more sensitive gel system that we used.

The same primers were then used for amplification by LS-PCR. The specific band, of approximately 290 bp, was found to be present as the most intense component of the LS-PCR profiles of all the serovars listed in Table 1 together with a complex set of LSPs. Examples of the data are shown in Fig. 1B, in which lanes 1 to 7 and 8 to 15 contain amplifications undertaken on different days and run on separate gels. The LS-PCR profiles were found to be serovar specific in every case tested, with the exception of the *L. interrogans* serovars icterohaemorrhagiae and copenhageni, the profiles of which were identical. This observation is in agreement with the results of ribotyping and total DNA restriction, which also found these serovars to be indistinguishable (8, 13). It is noteworthy that the two serovars produce the same severe life-threatening clinical form of the disease and use the same reservoir host. Several of the other serovars used were also very closely related but yielded distinct LS-PCR profiles, for example, *L. interrogans* serovars canicola and schueffneri, *L. kirschneri* serovars bafani and sumneri, and *L. biflexa* serovars patoc and andamana. It can be seen that very similar LS-PCR profiles are shared by the closely related pairs but that the details of the banding patterns serve to distinguish one from another in each case. One serovar, *L. interrogans* serovar canicola, was included twice to show the reproducibility of the LS-PCR analysis (Fig. 1B, lanes 5 and 9). Despite the slight differences in the intensities of the two gels and the length of time of electro-

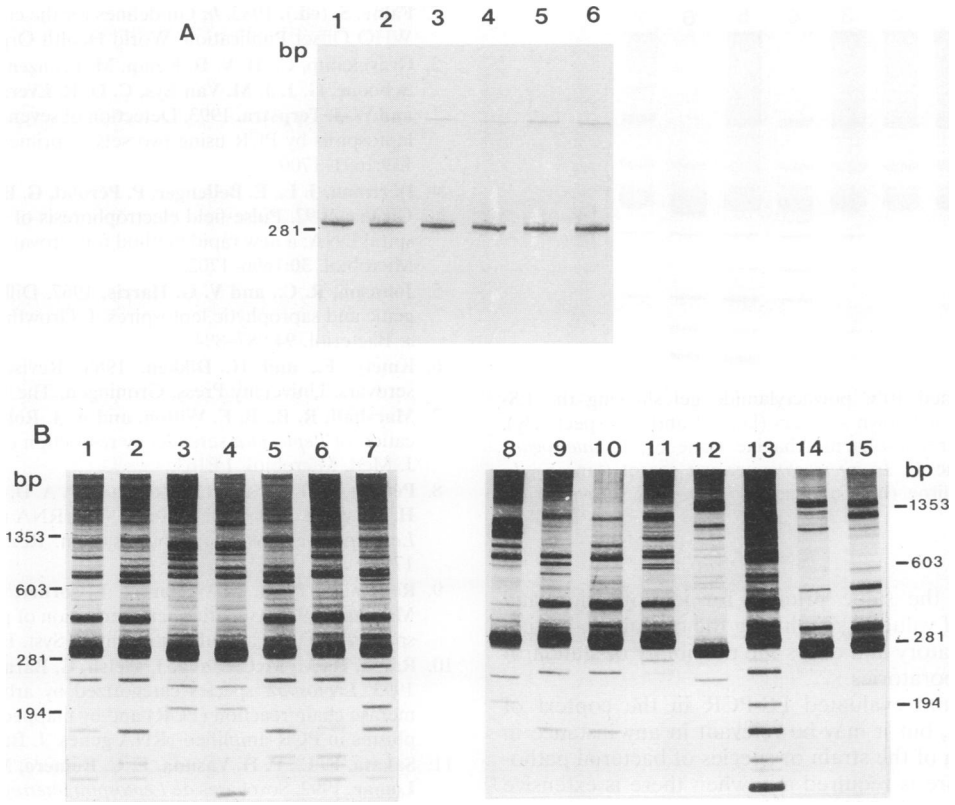

FIG. 1. (A) Silver-stained 10% polyacrylamide gel showing the specific PCR amplification products derived with primers G1-G2 and the following serovars: *L. interrogans* serovar pyrogenes (lane 1), *L. interrogans* serovar pomona (lane 2), *L. kirshneri* serovar grippotyphosa, (lane 3), *L. kirshneri* serovar cynopteri (lane 4), *L. interrogans* serovar canicola (lane 5), and *L. interrogans* serovar bataviae (lane 6). (B) Silver-stained 10% polyacrylamide gel showing the LS-PCR products derived using primers G1-G2 and the following serovars: *L. interrogans* serovar pyrogenes (lane 1), *L. interrogans* serovar pomona (lane 2), *L. kirshneri* serovar grippotyphosa (lane 3), *L. kirshneri* serovar cynopteri (lane 4), *L. interrogans* serovar canicola (lane 5), *L. interrogans* serovar bataviae (lane 6), *L. interrogans* sorovar australis (lane 7), *L. kirshneri* serovar bafani (lane 8), *L. interrogans* serovar canicola (lane 9), *L. interrogans* serovar schueffneri (lane 10), *L. kirshneri* serovar sumneri (lane 11), *L. interrogans* serovar djasiman (lane 12), *L. interrogans* serovar sentot (lane 13), *L. biflexa* sorovar patoc (lane 14), and *L. biflexa* sorovar andamana (lane 15).

phoresis, the same distinctive banding pattern is apparent in both cases.

The reproducibility and sensitivity of LS-PCR were further tested by using amounts of *Leptospira* DNA ranging from 5 ng to 32 fg, as shown in Fig. 2. Essentially the same complex pattern was evident in all tubes, although at lower concentrations some of the fainter bands were no longer visible. Nevertheless, adequate LSPs were obtained with as little as 160 fg, which corresponds to the DNA content of approximately 32 bacteria.

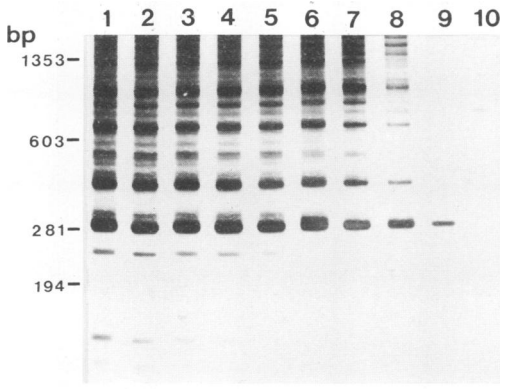

FIG. 2. Silver-stained 4% polyacrylamide gel showing the LS-PCR amplification products obtained with differents amounts of *L. interrogans* serovar bataviae DNA. Lanes 1 to 9, products resulting from 5 ng, 1 ng, 0.5 ng, 0.1 ng, 20 pg, 4 pg, 800 fg, 160 fg, and 32 fg of DNA, respectively; lane 10, a negative control (no DNA added).

To test the utility of the LS-PCR banding patterns for the identification of isolates, isolates of two unknown and recently isolated serovars (one from Rio de Janeiro and one from Belo Horizonte) were compared with a battery of reference strains (Fig. 3). The two isolates produced identical LS-PCR profiles and were identified as being of the icterohaemorrhagiae or copenhageni serovars (as noted above, these two serovars are indistinguishable by DNA-based methodologies). One of the two isolates (from Rio de Janeiro) was subsequently confirmed as being of the icterohaemorrhagiae serovar by traditional serology at the Institute Pasteur, Paris.

The proposed LS-PCR methodology combines the advantages of classical PCR and AP-PCR. It requires extremely low numbers of the organism, provides a positive internal control for the DNA being tested (on the basis of the specificities of the primers), and allows variants to be detected because of the polymorphisms of the LSPs. It offers a potential means of applying AP-PCR technology to the clinical laboratory, which is otherwise complicated by its extreme variability and promis-

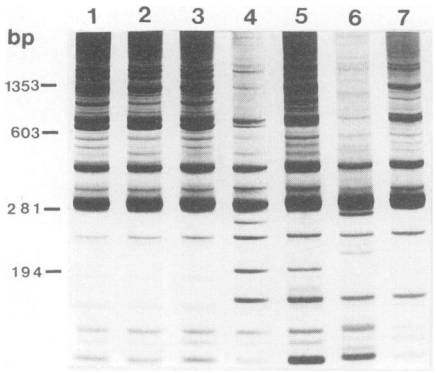

FIG. 3. Silver-stained 10% polyacrylamide gel showing the LS-PCR products of two unknown serovars (lanes 1 and 2, respectively), *L. interrogans* serovar icterohaemorrhagiae (lane 3), *L. interrogans* sorovar australis (lane 4), *L. interrogans* serovar sentot (lane 5), *L. interrogans* serovar sejroe (lane 6), and *L. interrogans* serovar pyrogenes (lane 7).

cuity. The use of the same primers for identification and diagnosis may be of value in simplifying the overall evaluation program of a laboratory and offers the possibility of standardization between laboratories.

We developed and evaluated LS-PCR in the context of *Leptospira* serovars, but it may be relevant in any instance in which identification of the strain or species of bacterial pathogen following culture is required and when there is extensive variation of the genomic DNA in question.

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