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IS*1533*-Based PCR Assay for Identification of *Leptospira interrogans* Sensu Lato Serovars

RICHARD L. ZUERNER,* DAVID ALT, AND CAROLE A. BOLIN

Leptospirosis and Mycobacteriosis Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010

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A PCR-based assay was developed for typing *L. interrogans* sensu lato serovars. The assay is designed to exploit the presence of many copies of the leptospiral insertion sequence IS*1533* and IS*1533*-like sequences present in the genomes of most leptospiral serovars. The PCR primers were designed to amplify DNA of unknown sequence between closely placed IS*1533* or IS*1533*-like sequences. Amplification reactions primed with IS*1533*-based primers generated products of different sizes. When few copies of IS*1533* were present in the genome, amplification of a few products was still detected. These results suggest that IS*1533* elements may be found close together. Analysis of DNA amplified from different serovars showed the presence of differently sized products, thus enabling the serovars to be identified. Genetic variation among isolates within the same serovar was also demonstrated with the IS*1533*-based primers. Amplification reactions using DNA extracted from the urine of infected animals generated specific products which were similar to the products generated from purified bacterial DNA. These results demonstrate that this assay is selective enough to be used for typing leptospiral serovars from clinical material and thus allows leptospiral typing without isolation of the bacteria in pure culture.

Leptospirosis is one of the most widespread zoonotic diseases in the world. Leptospirosis is a common disease in wildlife and livestock, where it can also be a source of human infection. Infections in livestock cause serious economic loss. The etiological agent of leptospirosis, *Leptospira interrogans* sensu lato, is a genetically diverse group of bacteria. Recent taxonomic analyses have led to the generation of several new species within *L. interrogans* sensu lato: *Leptospira borgpetersenii*, *Leptospira inadia*, *Leptospira interrogans* sensu stricto, *Leptospira kirschneri*, *Leptospira noguchii*, *Leptospira santarosai*, and *Leptospira weilii* (19, 30). Substantial antigenic variation also occurs within *L. interrogans* sensu lato, with antigenically indistinct isolates belonging to different species (e.g., serovar hardjo is found in both *L. interrogans* sensu stricto and *L. borgpetersenii* [21]).

A rapid and accurate method for typing leptospires from clinical samples is essential for controlling leptospirosis. Locating the source of infection is critical. However, the clinical manifestations of disease are similar for disease caused by different *Leptospira* species or serovars. Typically a given serovar develops a chronic, asymptomatic infection within a specific animal host species, providing a source of infection for other animals (12). Infection of a different species with that same serovar often results in a severe acute infection. Especially in humans, a severe infection can be caused by serovars commonly infecting wildlife or livestock. Thus, determining the source of infection on the basis of clinical signs of disease is not practical.

Existing methods for typing leptospiral isolates are slow and are often difficult to perform. Traditional serological typing of leptospiral isolates is a difficult and labor-intensive process involving the use of cross-adsorption agglutination reactions (2), and variation among laboratories is often seen. Restriction endonuclease analysis (REA) is useful, since nearly every serovar yields a unique digestion pattern (10, 22, 24, 34, 35). Discrimination among genetically related serovars by REA can be further improved by Southern blot analysis (24, 34, 35). We found previously that use of probes from a leptospiral insertion sequence, IS*1533*, is well suited for differentiating serovars on the basis of restriction fragment length polymorphisms (34, 35). Recently, arbitrarily primed PCR methodology has been used as an alternative approach to typing leptospiral isolates (15, 18). This approach yields a complex pattern of amplification products which differ from serovar to serovar, thus offering an alternative to REA for serovar identification.

A critical weakness common to all existing leptospiral typing methods is the need to obtain bacterial isolates in pure culture. Growth of leptospires, particularly from clinical isolates, is a slow and labor-intensive process and is often unreliable, even under ideal conditions. Thus, typing techniques dependent upon culture are likewise limited.

In this study, we developed a method which enables the infecting leptospiral serovar to be determined quickly and accurately by direct analysis of the urine of infected animals. The PCR assay uses primers directed to IS*1533*, copies of which we showed previously are widely distributed among *L. interrogans* sensu lato serovars and which are distributed differently in different serovars (34, 35). The present research expands on these earlier studies by using PCR to amplify segments of the leptospiral genome between closely placed copies of IS*1533* or IS*1533*-like sequences. Unique patterns of amplification products were detected for different serovars.

MATERIALS AND METHODS

Bacterial strains. *Leptospira* strains were propagated in EMJH medium at 30°C (4, 8). Strains used in this study are listed in Table 1.

Preparation of DNA and PCR techniques. Genomic DNA for restriction digestion and for PCR was extracted from *L. interrogans* as described by Theirmann et al. (22). Primers for PCR were as follows: EPR-2, 5′-CTC-GCA-TCT-AAC-CCA-CGT-TT-3′ (starting 14 bp from the left inverted repeat [IR]); and EPL-2, 5′-AGA-TTT-ACT-GCT-CCG-GAT-GG-3′ (starting 1 bp inside the

* Corresponding author. Mailing address: Leptospirosis and Mycobacteriosis Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 70, Ames, IA 50010. Phone: (515) 239-8392. Fax: (515) 239-8458. Electronic mail address: rzuerner@asrr.arsusda.gov.

TABLE 1. *L. interrogans* strains used in this study[a]

Serogroup	Serovar	Strain(s)
Australis	muenchen	Munchen c-90
Autumnalis	fort bragg	fort bragg
Bataviae	paidjan	paidjan
Canicola	broomi	patane
Celledoni	celledoni	celledoni
Djasiman	gurungi	gurung
Gryppotyphosa	canalzonae	cz188
Icterohaemorrhagiae	birkini	Birkin
Javanica	flumininse	Aa3
Pomona	tropica	Ca299U
Pyrogenes	camlo	LT64-47
Sarmin	rio	Rr5
Sejroe	hardjo	033 (1),[b] 203 (2), 057 (4), 067 (12), 197 (13), and 068 (14)
	istrica	bratislava
	polonica	493 Poland
	romanica	LM294
	sejroe	M84
	wolffi	3705
Tarrasovi	guidae	RP29
	moldaviae	114-2
	tarassovi	perepelicin
	tunis	P2/65

[a] Strains are part of the collection of the Leptospirosis Reference Center, National Animal Disease Center.

[b] All hardjo strains listed are *L. borgpetersenii*. The numbers in parentheses are genetic groups based on IS typing (35).

right IR) (32). The estimated melting temperature for these primers is about 70°C. PCRs were carried out in either a DNA Thermal Cycler or a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Conn.). Reaction mixes were prepared according to the manufacturer's directions (Perkin-Elmer). In the DNA Thermal Cycler, the reactions were carried out for 30 cycles of 1 min of denaturation (94°C), 2 min of annealing (65, 60, or 50°C), and 3 min of extension (72°C) with the extension time increased in 10-s increments per cycle starting after the sixth cycle. For detection of leptospiral DNA extracted from cattle urine, both control (in vitro-grown bacteria) and test samples were subjected to a modified protocol in which the annealing reaction time was increased to 3 min. Reactions were modified for use in the PCR System 9600 by altering the cycle times to 30 s of denaturation, 30 s of annealing, and 90 s of extension, with the extension time increased in 10-s increments per cycle starting after the sixth cycle.

Total DNA was extracted from urine of cattle experimentally infected with *L. borgpetersenii* serovar hardjo (1), by a method described elsewhere (26). Briefly, approximately 40 ml of urine was collected 15 min after an intravenous injection of 500 mg of furosemide. Bacteria were concentrated from each urine sample by centrifugation (16,000 × *g* for 30 min), and the pellet was washed once with 1 mM EDTA. The resulting pellet was suspended in 1.5 ml of distilled water and boiled for 1 min at 100°C.

Cloning and analysis of PCR products. PCR-amplified products were separated by continuous-field gel electrophoresis, stained, and visualized as described elsewhere (34). Fractionated DNA was transferred to Hybond-N and used for hybridization analysis. DNA amplification products were cloned into pCRII by using a kit from Invitrogen Corp. (San Diego, Calif.). Plasmid DNA was extracted as described elsewhere (34). Selected plasmids were used as templates for probe synthesis by nick translation with [α-^{32}P]dATP (600 Ci/mmol; ICN Pharmaceuticals, Irvine, Calif.) by using a kit from GIBCO-BRL (Gaithersburg, Md.). DNA immobilized on Hybond-N by UV cross-linking was hybridized with radioactive probes and washed as described elsewhere (34).

RESULTS

Primer selection rationale. IS*1533* is present in approximately 40 copies per genome of *L. borgpetersenii* serovar hardjo, the serovar from which this insertion sequence (IS) was originally isolated (33). The copy number of IS*1533*-like sequences ranges from 0 to approximately 60 copies per genome in different *L. interrogans* sensu lato serovars (34). Although the IS*1533* copies are well distributed in the genome, it was hypothesized that some IS*1533* sequences may be positioned close together. On the basis of this hypothesis, primers for PCR were designed from the IS*1533* sequence (32) so that they were oriented outward from the IS element. These primers started just inside the 5′ region of the "unique" region of IS*1533* (EPR-2) and in the 3′ IR (EPL-2). Amplification with these primers was expected to pass between two sets of IS*1533* or IS*1533*-like sequences in either direct-repeat or IR orientation (Fig. 1). Thus, a single primer could direct amplification of DNA between two sets of sequences in inverted orientation, while reactions using both primers would be needed to detect two sets of IS*1533* or IS*1533*-like sequences positioned as a direct repeat.

Amplification of *L. borgpetersenii* serovar hardjo DNA. Because IS*1533* was originally isolated from *L. borgpetersenii* serovar hardjo, initial studies used a homologous genetic background for developing the method. Each primer or primer pair directed the amplification of several fragments from *L. borgpetersenii* serovar hardjo genomic DNA (Fig. 2). The influence of annealing temperature was also tested by comparing amplifications at 65 (melting temperature [T_m] − 5°C) (data not shown), 60 (T_m − 10°C) (Fig. 2A), and 50°C (T_m − 20°C) (Fig. 2B). The amplification products generated from 65 and 60°C annealing temperatures were identical (data not shown). At the 50°C annealing temperature, more amplification products were seen, which suggests that some of these products result from priming from templates containing some degeneracy.

The relative levels of amplification products generated with

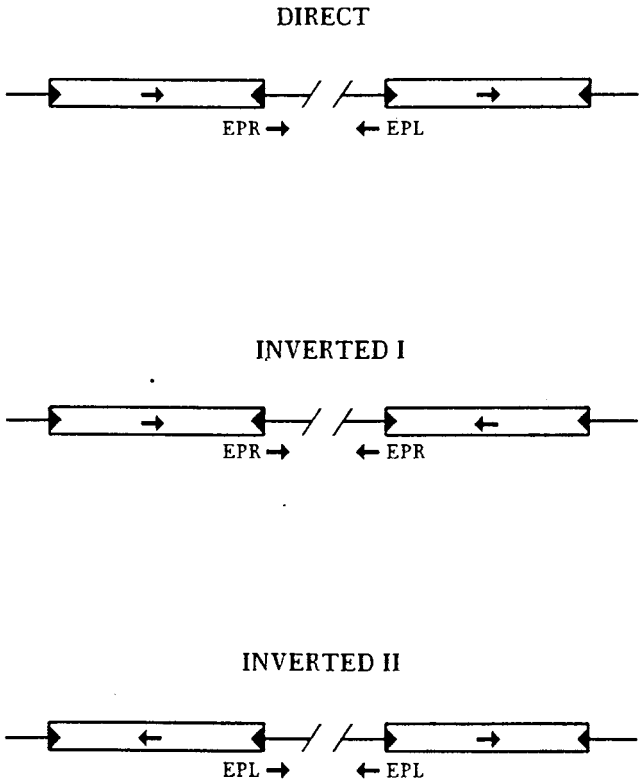

FIG. 1. Schematic of amplification strategy. IS*1533* is shown as blocks with the terminal IRs as triangles. Orientations for both direct repeats and IRs of IS*1533* are shown. The directions of primers used for amplification reactions are shown.

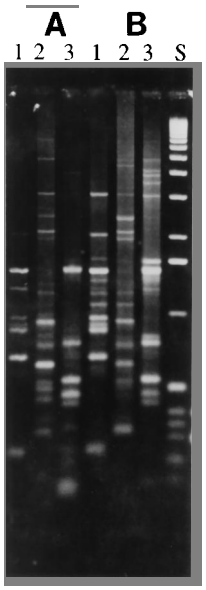

FIG. 2. Amplification of DNA from *L. borgpetersenii* serovar hardjo. DNA from *L. borgpetersenii* serovar hardjo strain 033 was amplified with primers EPR-2 (lanes 1), EPL-2 (lanes 2), or EPR-2–EPL-2 (lanes 3), with annealing temperatures of 60 (A) and 50°C (B). The amplification products were separated by agarose gel electrophoresis and visualized by UV illumination of the ethidium bromide-stained gel. Lane S contains size standards.

either EPR-2 or EPL-2 were altered when both primers were used together, resulting in a pattern different from simply a composite of the patterns produced by reactions primed with the individual primers (Fig. 2). To better evaluate the amplification reaction, several amplification products were cloned and analyzed. Hybridization analysis with cloned amplification products from reactions primed with EPR-2 alone, EPL-2 alone, or the EPR-2–EPL-2 primer pair showed that reaction mixtures primed with EPR-2 plus EPL-2 contained some products present in the reaction mixtures primed with just one primer and some unique products which would be expected by priming with both primers (data not shown). However, the complete amplification products were unstable in *Escherichia coli* during the initial cloning process since all had undergone deletions of one of the IRs (data not shown). Attempts to stabilize these sequences in a *recA* mutant host were unsuccessful.

Differentiation of *L. borgpetersenii* serovar hardjo isolates. Isolates of *L. borgpetersenii* serovar hardjo show genetic heterogeneity and fall into 15 discrete genetic groups when typed by REA and Southern blot analysis with IS*1533*-based probes (reference 35 and data not shown). Since the IS*1533*-based primers directed the synthesis of several different amplification products from DNA of the reference strain, 033, the possibility that these primers could also detect differences among isolates was tested. Genomic DNAs from isolates representing the different genetic groups of *L. borgpetersenii* serovar hardjo were used as templates for PCR with EPR-2, EPL-2, and the EPR-2–EPL-2 primer pair. The resulting amplification products allowed the isolates to be clustered into five different groups (Fig. 3) consistent with, but less specific than, the previously defined genetic groups (35). We have noted that there were some differences in the patterns of amplification reactions generated from different preparations of DNA extracted from the same isolates.

Applicability of IS*1533*-based PCR to other leptospiral serogroups. Previously we showed that IS*1533* or IS*1533*-like sequences were present in most *L. interrogans* sensu lato serovars (34). The EPR-2–EPL-2 primer combination was used to prime amplification reactions using DNA from serovar type strains held at the U.S. Department of Agriculture Leptospirosis Reference Center, National Animal Disease Center, Ames, Iowa. Amplification products were generated from DNA from 177 of the 182 pathogenic serovars composing the collection. Amplification products were also generated from *Leptospira biflexa* serovar patoc and *Leptonema illini* DNA, but no amplification products were generated from *Serpulina hyodysenteriae*, *Serpulina innocens*, or *Treponema phagedenis* DNA (data not shown). For each serovar for which amplification products were generated, a unique pattern was generated either with the EPR-2–EPL-2 primer pair or with EPL-2 alone, thus indicating that the technique was useful for typing different serovars. Examples of these results are shown in Fig. 4 and 5 and are described below.

Four members of *L. borgpetersenii* serogroup Tarrasovi (serovars tarrasovi, tunis, guidae, and moldaviae) are nearly indistinguishable by REA but have different hybridization patterns with IS*1533* probes (34). Because of the difficulty in resolving these serovars by REA, they were selected to test the applicability of the PCR assay for typing closely related serovars. The products of reactions primed with EPL-2, the EPL-2–EPR-2 combination, and EPR-2 allowed each serovar to be identified (Fig. 4).

An example of the breadth of positive PCRs is shown in Fig. 5. In this experiment, DNA samples representing several different *L. interrogans* sensu lato serogroups were amplified with the EPL-2–EPR-2 primer pair. Several examples of serovars within serogroup Sejroe were included to show the diversity within a given serogroup (Fig. 5, lanes 4 to 8). In addition to that of the serogroups shown in Fig. 5, DNA from representatives of all recognized *L. interrogans* sensu lato serogroups was amplified (data not shown). Additionally, IS*1533*-primed products were detected by using DNA from serovars spanning all of the newly recognized species previously classified as *L. interrogans* sensu lato.

Detection of *L. interrogans* DNA in urine of infected livestock. Total DNA was extracted from the urine of cattle experimentally infected with two different genetic types of *L.*

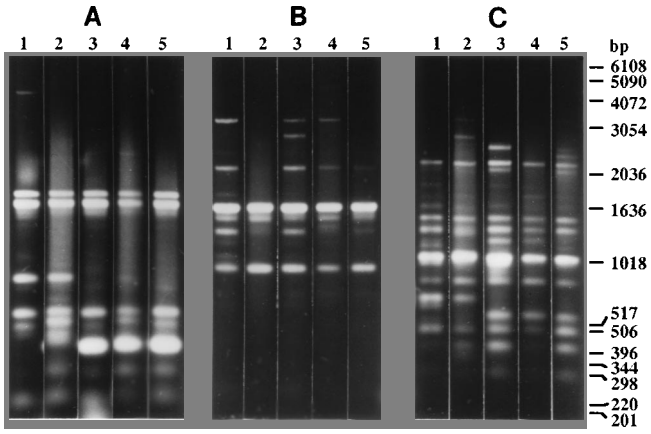

FIG. 3. Comparison of *L. borgpetersenii* serovar hardjo isolates. DNA from serovar hardjo strains 203 (lanes 1), 67 (lanes 2), 197 (lanes 3), 75 (lanes 4), and 68 (lanes 5) was amplified with primers EPR-2–EPL-2 (A), EPR-2 (B), and EPL-2 (C) at a 50°C annealing temperature. The amplification products were separated by agarose gel electrophoresis and visualized by UV illumination of the ethidium bromide-stained gel.

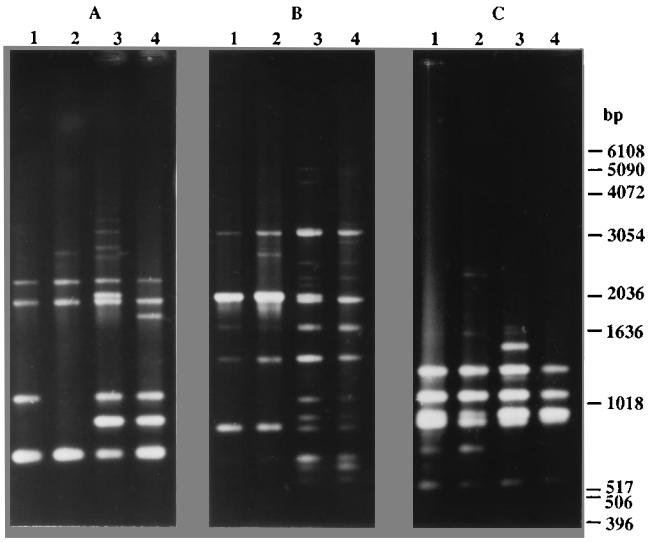

FIG. 4. Analysis of different *L. borgpetersenii* serovars. The amplification products from reactions with mixtures containing *L. borgpetersenii* serogroup Tarrasovi serovars tarrasovi (lanes 1), guidae (lanes 2), tunis (lanes 3), and moldaviae (lanes 4) and primed with EPR-2–EPL-2 (A), EPR-2 (B), and EPL-2 (C) were separated by agarose gel electrophoresis and visualized by UV illumination of the ethidium bromide-stained gel.

borgpetersenii serovar hardjo (strains 197 and 203). The extracted DNA was amplified with the EPR-2–EPL-2 primer pair. The pattern of amplification products was nearly identical to the pattern of products generated with target DNA from in vitro-grown bacteria and was sufficient for determining the genetic type of *L. borgpetersenii* serovar hardjo used to infect the animal (Fig. 6).

DISCUSSION

The methods described in this report enable *L. interrogans* serovars to be typed directly from the urine of infected animals. Specific amplification of *L. interrogans* DNA with outward-facing primers based on IS*1533* sequences generated several differently sized products. Because the electrophoretic patterns of these amplification products are characteristic for each serovar, the patterns can be used to differentiate among serovars and, to some degree, among genetic variants within the same serovar. The technique described here circumvents the need for bacteriological culture of isolates, which is a critical and unreliable step upon which other published leptospiral typing methods are dependent.

Typically, a given serovar is found associated with a specific animal host species. Development of a rapid typing system for *L. interrogans* serovars should make it possible to quickly determine potential sources of infection during an outbreak and thus improve control of the disease. Additionally, because growth of *L. interrogans* isolates is often difficult, the PCR method described here may assist in the development of more-effective vaccination strategies by determining the prevalence of different serovars, perhaps in the absence of positive culture results.

Leptospira strains are noted for variability in genomic organization (5, 6, 36) and repetitive DNA content (13, 17, 24, 29, 31–34). Unique or characteristic restriction fragment length polymorphisms are seen for each serovar and are associated with different classes of repetitive DNA, including IS elements (34). This aspect of the leptospiral genome was exploited in the development of the PCR-based technique described here. As with REA and Southern blot typing methods, we found that variation in the amplification products generated with the IS*1533*-based primers allows serovars to be identified. The resolution of the technique extends to variation within the same serovar. Because (at least with serovar hardjo) genetically different groups occur in different geographical locations (35), it may be possible to localize outbreaks to imported or newly transported livestock. We note that although the PCR technique described here is not as sensitive in detecting genetic variation among different serovar hardjo isolates as Southern blot analysis is (PCR detects 5 groups while hybridization resolves 15 different groups [35]), it is much more rapid and easier to perform and can be done with clinical samples (Fig. 6). This is of particular importance in studying serovar hardjo isolates, for primary isolation can take more than 6 months before sufficient growth is obtained for typing and isolation from vaccinated, yet infected, animals is very difficult.

Using hybridization techniques, we showed that IS*1533* or IS*1533*-like sequences are widely distributed among many *L. interrogans* sensu lato serovars (34). Although previous studies failed to detected IS*1533*-like sequences in the saprophytic species *L. biflexa* or *Leptonema illini* during hybridization studies (33), a small number of amplification products are generated from DNA of these species with IS*1533*-based primers. Evolutionary relationships derived by 16S rRNA sequence comparisons show that the genera *Leptospira* and *Leptonema* are closely related (14). The data presented here suggest that IS*1533*-like sequences are well distributed among members of the genera *Leptospira* and *Leptonema* but are not seen among

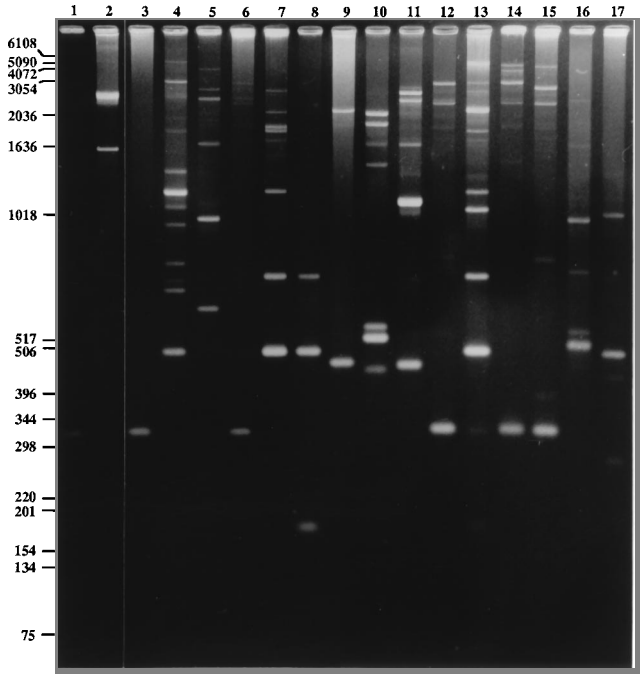

FIG. 5. Analysis of different *L. interrogans* sensu lato serovars. DNA representing serogroups Australis (lane 1), Autumnalis (lane 2), Djasiman (lane 3), Sejroe (lanes 4 to 8) (serovars istrica [lane 4], wolffi [lane 5], romanica [lane 6], sejroe [lane 7], and polonica [lane 8]), Sarmin (lane 9), Celledoni (lane 10), Grippotyphosa (lane 11), Pyrogenes (lane 12), Bataviae (lane 13), Canicola (lane 14), Pomona (lane 15), Javanica (lane 16), and Icterohaemorrhagiae (lane 17) was amplified with the EPR-2–EPL-2 primer pair. The amplification products were separated by agarose gel electrophoresis and visualized by UV illumination of the ethidium bromide-stained gel. Numbers on the left indicate molecular size in base pairs.

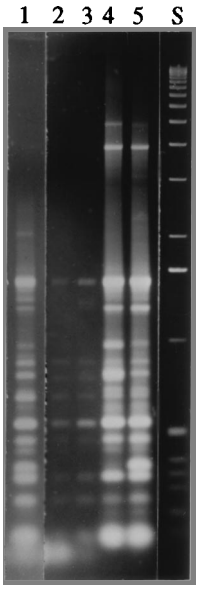

FIG. 6. Detection of leptospiral DNA from infected cattle. Shown is DNA extracted from urine obtained from cattle experimentally infected with *L. borgpetersenii* serovar hardjo strain 197 (lane 1) or strain 203 (lanes 2 and 3) and purified DNA extracted from strain 203 (lane 4) or strain 197 (lane 5) cells grown in vitro. Amplification was with the EPR-2–EPL-2 primer pair. Annealing was at 60°C for 3 min. The amplification products were separated by agarose gel electrophoresis and visualized by UV illumination of the ethidium bromide-stained gel. Lane S contains size standards.

other spirochetes. Thus, the amplification reactions described here are useful for selectively amplifying leptospiral DNA, and because the patterns of products are serovar specific, the assay provides an easy typing system.

Previous PCR-based approaches for leptospiral detection have used sequences from rRNA (7, 11), periplasmic flagellum genes (28), or sequences of unknown function (23). Although diagnostic tests based on these target sequences are sensitive, they offer limited ability to differentiate serovars (20, 28). As an alternative approach, arbitrarily primed PCR, which provides a sensitive method for serovar identification, has been used for differentiating leptospiral serovars (15, 18). However, for accurate arbitrarily primed PCR the target DNA should be prepared from pure cultures, because contaminating DNA from other sources, such as host animal tissue, can contribute to the amplification products. In this report we show that *L. interrogans* can be detected and typed on the basis of amplifying DNA extracted from urine of experimentally infected livestock. Although the most prominent amplification products in samples of DNA from in vitro-grown bacteria were the same as those from DNA samples from in vivo-grown bacteria, some of the less prominent bands were not detected. These differences may be attributed to degradation of leptospiral DNA extracted from urine. The DNA extracted from in vitro-grown leptospiral cells in late-logarithmic-phase or stationary-phase growth shows evidence for significant degradation (data not shown). This may also account for some of the minor differences in the amplification products generated with independent DNA extractions from the same isolate. It is important to note that no new amplification products are detected in clinical samples and that noninfected animals yield no detectable amplification products (data not shown), which shows that this technique is specific for leptospiral DNA.

The strategy of the PCR method described here is similar to that of methods reported for typing other eubacteria by using repetitive extragenic palindromic (REP) sequences (3, 25). The REP sequences are short nucleotide sequences (<150 bp in length) present in high copy number within the genomes of gram-negative bacteria. By using primers homologous to these sequences, *Bradyrhizobium* sp. (9), *Citrobacter diversus* (27), and *Rhizobium* sp. (3) isolates can be distinguished. Presumably the amplification products result from amplification from one REP sequence to the next. With the method described here, we find that PCR amplification outward from IS*1533* sequences can produce several products which vary in size and number depending on the serovar. The more closely related the serovars, the more similar the patterns of PCR products appear (Fig. 3 and 4A). Initial nucleotide sequence analysis of the cloned PCR products indicates that these products contain IS*1533* sequences and flanking DNA. Thus, different copies of IS*1533*-like sequences are closely placed in leptospiral genomes.

We predict that the strategy for typing bacterial strains described here should be generally applicable, since other IS elements are found in high copy number and reside in polymorphic genomic regions. Studies with the *Mycobacterium tuberculosis* element IS*981* also detect variation among isolates (16). In addition, the presence of some IS elements at high copy number in other bacteria, such as IS*1* in *Shigella* spp. (ca. 200 copies per genome), suggests that IS-based PCR assay typing schemes similar to that described here could work with other genera.

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