IDENTIFICATION AND TYPING OF BACTERIA

Random amplified polymorphic DNA fingerprinting for rapid identification of leptospiros of serogroup Sejroe

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Summary. Eight leptospiral and three non-leptospiral primers were examined in the development of a random amplified polymorphic DNA (RAPD) fingerprinting method for identification of leptospiral serovars within the serogroup Sejroe. The strains examined comprised 19 reference strains of serogroup Sejroe, five field strains and 17 *Leptospira borgpetersenii* serovar *hardjo* type hardjobovis strains. Unique DNA banding patterns were obtained for each of the 17 reference strains with *L. hardjo* type hardjobovis-derived primers, and all clinical isolates were identified as *L. hardjo* type hardjobovis. Although the profiles were specific for each serovar, most serovars within serogroup Sejroe clustered into previously recognised genetic groups. RAPD fingerprinting is a simple, rapid method suitable for the identification of some *Leptospira* serovars.

Introduction

Traditionally, leptospiros are classified and identified in serological techniques such as the cross-absorption test.^{1,2} However, these techniques are laborious and show poor inter-laboratory reproducibility. Monoclonal antibody methods are more rapid and specific,^{3,4} but still cannot distinguish all serovars. DNA-based methods for identification of leptospiros include restriction fragment length polymorphism (RFLP) analysis,^{5–8} DNA-DNA hybridisation,^{9–15} pulsed field gel electrophoresis (PFGE),¹⁶ polymerase chain reaction (PCR) followed by RFLP analysis^{17,18} and random amplified polymorphic DNA (RAPD) fingerprinting.^{17,19} RFLP analysis is generally more sensitive and discriminatory than serotyping, but some serovars are not readily distinguished²⁰ and the complex banding patterns are difficult to interpret. Hybridisation of labelled DNA probes to RFLP blots greatly simplifies the visualised banding patterns facilitating interpretation,¹² and allows serovars with similar RFLP banding patterns to be differentiated.¹⁴ On the evidence of DNA-DNA hybridisation, Yasuda *et al.*¹³ proposed 10 *Leptospira* species comprising the six pathogenic species *L. interrogans*, *L. weilii*, *L. borgpetersenii*, *L. noguchii*, *L. santarosai* and *L. inadai*, and the four saprophytic species *L. meyeri*, *L. wolbachii*, *L. biflexa* and *L. parva*. Ramadass *et al.*¹¹ found a seventh group of pathogenic strains designated *L. kirschnerii*.

RFLP analysis, DNA-DNA hybridisation and

PFGE are slow and require large amounts of purified DNA. In contrast, PCR-based methods are rapid and require only small amounts of DNA.^{21–24} PCR-based fingerprinting systems have been developed for a range of bacteria.^{17,19,25–28} Ralph *et al.*¹⁷ categorised *Leptospira* serovars from various serogroups by arbitrarily primed PCR, and by restriction polymorphisms of PCR-amplified rRNA genes. Corney *et al.*¹⁹ discriminated between nine reference strains representing nine serovars of seven *Leptospira* serogroups and identified 26 isolates from Australian cattle with an RAPD fingerprint method based on non-leptospiral primers.

This study investigated the use of eight leptospiral and three non-leptospiral primers in RAPD fingerprinting of leptospiros.

Materials and methods

Bacterial strains and preparation of chromosomal DNA

Reference strains (table I) of serogroup Sejroe (obtained from C. Gravekamp) and 21 *L. hardjo* subtype hardjobovis isolates (table II)^{29–31} were examined. Strains were cultured at 29°C in EMJH medium³² to a density of *c.* 10⁸ cells/ml and centrifuged at 12000 *g* for 30 min at 4°C. Pellets were washed twice in phosphate-buffered saline (pH 7.4) and resuspended in lysis buffer (50 mM NaCl, 10 mM EDTA, in 50 mM Tris-HCl buffer, pH 7.4). After addition of sodium dodecyl sulphate 0.5% and pronase (Sigma) 20 µg/ml the suspension was incu-

Table I. Reference strains investigated

RAPD Group	Serovar	Genomic species
I	<i>hardjo</i> subtype hardjobovis	<i>borgpetersenii</i> ^{15, 17, 19, 20}
	<i>balcanica</i>	<i>borgpetersenii</i> ^{17, 19}
	<i>nyanza</i>	<i>borgpetersenii</i> ¹⁹
II	<i>dikeni</i>	<i>borgpetersenii</i> ^{15, 19}
	<i>polonica</i>	<i>borgpetersenii</i> ^{15, 19}
	<i>sejroe</i>	<i>borgpetersenii</i> ^{19, 20}
	<i>istrica</i>	<i>borgpetersenii</i> ^{15, 19}
	<i>saxkoebing</i>	<i>borgpetersenii</i> ^{17, 19, 24} or <i>interrogans</i> ²⁰
III	<i>trinidad</i>	<i>santarosai</i> ^{15, 19}
	<i>gorgas</i>	<i>santarosai</i> ^{15, 19}
	<i>recreo</i>	<i>santarosai</i> ¹⁹ or <i>interrogans</i> ¹⁵
IV	<i>haemolytica</i>	<i>interrogans</i> ^{17, 19}
	<i>medanensis</i>	<i>interrogans</i> ^{15, 17, 19}
	<i>geyweera</i>	<i>interrogans</i> ^{15, 19}
	<i>roumanica</i>	<i>interrogans</i> ^{15, 19}
	<i>hardjo</i> subtype hardjoprajitno	<i>interrogans</i> ^{14, 17, 19}
	<i>wolfii</i>	<i>interrogans</i> ^{17, 20} or <i>borgpetersenii</i> ²¹
V	<i>caribe</i>	<i>santarosai</i> ^{15, 19}
VI	<i>guaricura</i>	<i>santarosai</i> ¹⁹

bated for 2 h at 60°C. Proteinase K (Sigma) 20 µg/ml was added and digestion was continued for a further 2 h at 37°C. DNA was extracted³³ and stored at 4°C.

Random amplified polymorphic DNA (RAPD) fingerprinting

PCR reaction mixtures contained chromosomal DNA 50 ng, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 4 mM MgCl₂, 0.1 mM of each of the four deoxy-nucleotide triphosphates (Pharmacia), 300 pM of each primer, 0.5 U *Taq* DNA polymerase (Boehringer) and gelatin 0.01 % w/v. The mixtures were covered with 50 µl of mineral oil and placed in a PCR processor (Cetus). The first two cycles comprised denaturation for 5 min at 95°C, annealing of primers for 5 min at 40°C and extension for 5 min at 72°C. The subsequent 35 cycles comprised denaturation for 1 min at 95°C, annealing of primers for 1 min at 60°C and extension for 3 min at 72°C, with extension for 10 min during the last cycle. PCR products were analysed by electrophoresis through non-denaturing polyacrylamide 17 × 14.5 × 0.1 cm 6 % gels at 160 V for 4 h.³³

Results

Of the 11 primers examined, only the leptospiral primer pair B11 and B12 (CCGGAAGAAGGGGCGCCAT and CGATTTAGAAGGACTTGACAC) produced banding patterns that were unique for each serovar (fig. 1, lanes 2–20). However, serovars *polonica*

Fig. 1. RAPD fingerprints of 19 *Leptospira* serovars within serogroup Sejroe. Lanes: 1 and 22, DNA ladder (PGEM7 digested by *Hpa*II); 2, *hardjo* subtype hardjobovis strain Sponselee; 3, *balcanica*; 4, *nyanza*; 5, *dikeni*; 6, *saxkoebing*; 7, *polonica*; 8, *sejroe*; 9, *istrica*; 10, *trinidad*; 11, *gorgas*; 12, *caribe*; 13, *recreo*; 14, *haemolytica*; 15, *medanensis*; 16, *geyweera*; 17, *roumanica*; 18, *guaricura*; 19, *wolfii*; 20, *hardjo* subtype hardjoprajitno; 21, negative control.

Table II. *L. hardjo* subtype hardjobovis isolates investigated

Strain	Origin
Sponselee	bovine; Netherlands*
Mulder	bovine; Netherlands†
Bakker	bovine; Netherlands†
2034	bovine; Netherlands†
25	sheep; Netherlands†
146	bovine; Ireland§
147	bovine; Ireland§
003	bovine; Nebraska, USA‡
033	bovine; Colorado, USA‡
272	bovine; Chile‡
057	bovine; Italy‡
196	bovine; Switzerland‡
145	bovine; Ireland‡
323	bovine; Ireland‡
051	bovine; Iowa, USA‡
328	bovine; Switzerland‡
330	bovine; Portugal‡
332	bovine; New Zealand‡
067	bovine; Florida, USA‡
197	bovine; Nebraska, USA‡
063	bovine; Israel‡

*Reference strain provided by C. Gravekamp (WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis, Amsterdam).

†Isolates from our own collection (see references 29–31).

‡Isolates provided by C. A. Bolin (National Animals Disease Centre, Ames, Iowa).

§Isolates provided by W. Ellis (Department of Agriculture, Belfast).

Fig. 2. RAPD fingerprints of *L. hardjo* strains. Lanes: 1, negative control; 2, *hardjo* subtype hardjoprajitno; 3, strain Sponselee; 4, strain Mulder; 5, strain Bakker; 6, strain 2034; 7, strain 25; 8, strain 328; 9 and 10 represent profiles found among the remainder of strains donated by C. A. Bolin and W. Ellis; 11, DNA ladder (PGEM7 digested by *Hpa*II). Arrows indicate the small differences observed in the reference strain Sponselee (lane 3) and strain 328 (lane 8) in comparison to the other *hardjo* subtype hardjobovis strains.

and *istricea* could not be distinguished (fig. 1, lanes 7 and 9). Patterns obtained with concentrations of 4–6 mM $MgCl_2$ in the PCR reaction mixture were satisfactory, but banding patterns became non-serovar-specific at lower concentrations. Primer concentrations ≥ 100 pM increased the product yield without change in the banding pattern. At template DNA concentrations between 0.1 and 10 ng/ μ l of PCR reaction mixture there was no significant variation in banding profiles. Thermal cycling through 36 equal cycles of denaturation for 1 min at 94°C, annealing for 1 min at 27°C and extension for 3 min at 72°C did not improve the specificity or enlarge the diversity of banding patterns.

Visual analysis of the number of bands shared between profiles indicated that the serovars could be divided into four broad groups, with the *caribe* and *guaricura* serovars as two further, grossly dissimilar single member groups (table I). There were major differences in the banding patterns of the genetically distinct but serologically similar serovar *hardjo* type hardjobovis and type hardjoprajitno (fig. 1, lanes 2 and 20; fig. 2, lanes 2 and 3). As expected, most of the 21 *hardjo* type hardjobovis strains showed closely similar banding patterns (fig. 2, lanes 8–10), although they were of different geographical origin. The banding patterns of strains from our own collection were identical (fig. 2, lanes 4–7). All these isolates yielded fingerprints similar to those of the 17 *hardjo* type hardjobovis strains and the reference strain Sponselee. No differences in fingerprints were observed between *hardjo* type hardjobovis strains isolated from urine or kidney tissue of a single animal, nor in strains passaged in EMJH medium.

Discussion

RAPD is a simple rapid method for differentiation of *Leptospira* serovars within serogroup Sejroe, giving reproducible, discrete and serovar-specific banding patterns. Serovars *istricea* and *polonica* could not be distinguished from one another, but these are also indistinguishable in RFLP analysis and with recombinant DNA probes.¹² All the other serovars, including the serologically identical but genetically distinct serovars *L. hardjo* type hardjoprajitno and type hardjobovis, could be differentiated. In visual analysis of the RAPD banding patterns, most serovars clustered in the genetic groups described by Van Eys *et al.*¹² and others,^{10, 11, 13, 17, 24} indicating that RAPD may be useful in classification (table I).

Small differences between strains of serovar *hardjo* type hardjobovis were found in RAPD. Pacciarini *et al.*⁹ also found small genetic differences between the reference strain Sponselee and clinical isolates of serovar *hardjo* type hardjobovis with a repetitive sequence, cloned from *L. interrogans* serovar *hardjo* type hardjoprajitno, as a probe for serovar identification. Zuerner *et al.*¹⁵ found at least 14 genetic differences within various *hardjo* type hardjobovis

strains; this high level of discrimination may be explained by the high frequency of transposition of the insertion sequence detected by the probe.

After detection of an infection, a fast and accurate identification of the serovar involved is often necessary for diagnostic and epidemiological purposes. We conclude that RAPD fingerprinting is a simple, rapid non-isotopic method for identification of *Leptospira* serovars of serogroup Sejroe.

References

1. Cole JR, Sulzer CR, Pursell AR. Improved microtechnique for the leptospiral microscopic agglutination test. *Appl Microbiol* 1973; **25**: 976-980.
2. Wolff JW. The laboratory diagnosis of leptospirosis. Springfield, Charles Thomas. 1954: 99.
3. Kobayashi Y, Tamai T, Sada E *et al.* Antigenic analysis of serogroup icterohaemorrhagiae using monoclonal antibodies. *Isr J Vet Med* 1987; **43**: 277-287.
4. Terpstra WJ, Korver H, Van Leeuwen J, Klatser PR, Kolk AH. The classification of Sejroe group serovars of *Leptospira interrogans* with monoclonal antibodies. *Zentralbl Bakteriol Mikrobiol Hyg A* 1985; **259**: 498-506.
5. Ellis WA, Montgomery JM, Thiermann AB. Restriction endonuclease analysis as a taxonomic tool in the study of pig isolates belonging to the Australis serogroup of *Leptospira interrogans*. *J Clin Microbiol* 1991; **29**: 957-961.
6. Hookey JV, Waitkins SA, Gahir R *et al.* The use of bacterial restriction endonuclease analysis to classify icterohaemorrhagiae and autumnalis serovars. *Isr J Vet Med* 1987; **43**: 265-270.
7. Terpstra WJ, Korver H, Schoone GJ *et al.* Comparative classification of leptospira serovars of the Pomona group by monoclonal antibodies and restriction endonuclease analysis. *Zentralbl Bakteriol Mikrobiol Hyg A* 1978; **266**: 412-421.
8. Thiermann AB, Handsaker AL, Moseley SL, Kingscote B. New method for classification of Leptospiral isolates belonging to serogroup Pomona by restriction endonuclease analysis: Serovar *kennewicki*. *J Clin Microbiol* 1985; **21**: 585-587.
9. Pacciarini ML, Savio ML, Tagliabue S, Rossi C. Repetitive sequences cloned from *Leptospira interrogans* serovar *hardjo* genotype *hardjoprajitno* and their application to serovar identification. *J Clin Microbiol* 1992; **30**: 1243-1249.
10. Perolat P, Lecuyer I, Postic D, Baranton G. Diversity of ribosomal DNA fingerprints of *Leptospira* serovars provides a database for subtyping and species assignment. *Res Microbiol* 1993; **144**: 5-15.
11. Ramadass P, Jarvis BDW, Corner RJ, Penny D, Marshall RB. Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. *Int J Syst Bacteriol* 1992; **42**: 215-219.
12. Van Eys GJJM, Gerritsen MJ, Korver H, Schoone GJ, Kroon CC, Terpstra WJ. Characterization of serovars of the genus *Leptospira* by DNA hybridization with *hardjovovis* and *icterohaemorrhagiae* recombinant probes with special attention to serogroup Sejroe. *J Clin Microbiol* 1991; **29**: 1042-1048.
13. Yasuda PH, Steigerwalt AG, Sulzer KR, Kaufmann AF, Rogers F, Brenner DJ. Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. *Int J Syst Bacteriol* 1987; **37**: 407-415.
14. Zuerner RL, Bolin CA. Nucleic acid probe characterizes *Leptospira interrogans* serovars by restriction fragment length polymorphisms. *Vet Microbiol* 1990; **24**: 355-366.
15. Zuerner RL, Ellis WA, Bolin CA, Montgomery JM. Restriction fragment length polymorphisms distinguish *Leptospira borgpetersenii* serovar *hardjo* type *hardjo-bovis* isolates from different geographical locations. *J Clin Microbiol* 1993; **31**: 578-583.
16. Hermann JL, Bellenger E, Perolat P, Baranton G, Saint Girons I. Pulsed-field gel electrophoresis of *NotI* digests of leptospiral DNA: a new rapid method of serovar identification. *J Clin Microbiol* 1992; **30**: 1696-1702.
17. Ralph D, McClelland M, Welsh J, Baranton G, Perolat P. *Leptospira* species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. *J Bacteriol* 1993; **175**: 973-981.
18. Woodward MJ, Sullivan GJ, Palmer NMA, Woolley JC, Redstone JS. Development of a PCR test specific for *Leptospira hardjo* genotype *bovis*. *Vet Rec* 1991; **128**: 282-283.
19. Corney BG, Colley J, Djordjevic SP, Whittington R, Graham GC. Rapid identification of some *Leptospira* isolates from cattle by random amplified polymorphic DNA fingerprinting. *J Clin Microbiol* 1993; **31**: 2927-2932.
20. Thiermann AB, Handsaker AL, Foley JW, White FH, Kingscote BF. Reclassification of North American leptospiral isolates belonging to serogroups Mini and Sejroe by restriction endonuclease analysis. *Am J Vet Res* 1986; **47**: 61-66.
21. Van Eys GJJM, Gravekamp C, Gerritsen MJ *et al.* Detection of leptospires in urine by polymerase chain reaction. *J Clin Microbiol* 1989; **27**: 2258-2262.
22. Gerritsen MJ, Olyhoek T, Smits MA, Bokhout BA. Sample preparation method for polymerase chain reaction-based semiquantitative detection of *Leptospira interrogans* serovar *hardjo* subtype *hardjovovis* in bovine urine. *J Clin Microbiol* 1991; **29**: 2805-2808.
23. Gravekamp C, Van de Kemp H, Franzen M *et al.* Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *J Gen Microbiol* 1993; **139**: 1691-1700.
24. Mérien F, Amouriaux P, Perolat P, Baranton G, Saint Girons I. Polymerase chain reaction for the detection of *Leptospira* spp. in clinical samples. *J Clin Microbiol* 1992; **30**: 2219-2224.
25. Bassam BJ, Caetano-Anolles G, Gresshoff PM. DNA amplification fingerprinting of bacteria. *Appl Microbiol Biotechnol* 1992; **38**: 70-76.
26. Van Belkum A, Struelens M, Quint W. Typing of *Legionella pneumophila* strains by polymerase chain reaction-mediated DNA fingerprinting. *J Clin Microbiol* 1993; **31**: 2198-2200.
27. Giesendorf BAJ, van Belkum A, Koeken A *et al.* Development of species-specific DNA probes for *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* by polymerase chain reaction fingerprinting. *J Clin Microbiol* 1993; **31**: 1541-1546.
28. Woodward MJ, Redstone JS. Differentiation of leptospira serovars by the polymerase chain reaction and restriction fragment length polymorphism. *Vet Rec* 1993; **132**: 325-326.
29. Gerritsen MJ, Koopmans MJ, Olyhoek T. Effect of streptomycin treatment on the shedding of and the serologic responses to *Leptospira interrogans* serovar *hardjo* subtype *hardjovovis* in experimentally infected cows. *Vet Microbiol* 1993; **38**: 129-135.
30. Gerritsen MJ, Koopmans MJ, Dekker TC, De Jong MC, Moerman A, Olyhoek T. Effective treatment with dihydrostreptomycin of naturally infected cows shedding *Leptospira interrogans* serovar *hardjo* subtype *hardjovovis*. *Am J Vet Res* 1994; **55**: 339-343.
31. Gerritsen MJ, Koopmans MJ, Peterse DJ, Olyhoek T. Sheep as maintenance host for *Leptospira interrogans* serovar *hardjo* subtype *hardjovovis*. *Am J Vet Res* 1994; **55**: 1232-1237.
32. Johnson RC, Harris VG. Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. *J Bacteriol* 1967; **94**: 27-31.
33. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory. 1982.