J. Med. Microbiol. — Vol. 46 (1997), 927–932

MOLECULAR TAXONOMY AND EPIDEMIOLOGY

Simplified analysis of pathogenic leptospiral serovars by random amplified polymorphic DNA fingerprinting

B. G. CORNEY, JANE COLLEY* and G. C. GRAHAM†

*Rockhampton Veterinary Laboratory, Department of Primary Industries, Box 6014 Rockhampton Mail Centre, Queensland 4702, *Animal Research Institute, Department of Primary Industries Locked Mail Bag 4, Moorooka, Queensland 4105 and †Cooperative Centre for Tropical Pest Management, University of Queensland, St Lucia, Queensland 4072, Australia*

A rapid, simplified procedure combining random amplified polymorphic DNA (RAPD) fingerprinting of boiled cultures with high resolution agarose gel electrophoresis was used to compare strains from 46 pathogenic leptospiral serovars. The serovars were placed in eight groups on the basis of RAPD profile similarities. Groups 1–7 corresponded with the genome species *Leptospira interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L. kirschneri* and *L. meyeri*. The eighth group did not correspond with a known genome species and may represent a new genome species. Primer choice determined the degree of discrimination possible between closely related serovars and genotypes. This procedure, unlike other procedures used for analysing taxonomic relationships between leptospiral serovars, does not require extensive DNA purification, polyacrylamide gel electrophoresis or autoradiography.

Introduction

Random amplified polymorphic DNA (RAPD) fingerprinting [1], also known as arbitrarily primed polymerase chain reaction (AP-PCR) [2] is a polymerase chain reaction (PCR)-based DNA fingerprinting method. RAPD has been used to type isolates of a number of bacteria [3–6]. More recently RAPD was used to determine the taxonomic relationships between leptospiral serovars [7] and for typing bovine isolates of *Leptospira* [8]. Pérolat *et al.* [9] used AP-PCR to examine genetic variability among strains of *Leptospira* serovar hardjo.

Pathogenic serovars of the genus *Leptospira* are assigned to one of the genome species *L. interrogans, L. weilii, L. borgpetersenii, L. noguchii, L. santarosai, L. inadai* or *L. kirschneri* by DNA hybridisation [10, 11]. The non-pathogenic serovars are similarly distributed among several genome species [12]. Ralph *et al.* [7] used RAPD as a rapid method for classifying serovars. They performed RAPD on purified DNA and analysed the products by polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. This study demonstrates a simpler and more rapid method of examining the taxonomic relationships between serovars in which RAPD is performed on boiled cultures and the products are analysed by high resolution agarose gel electrophoresis. This procedure was used by Corney *et al.* [8] for serovar identification of bovine *Leptospira* isolates. It has none of the hazards associated with PAGE or autoradiography due to handling acrylamide or radioisotopes.

Received 30 Dec. 1996; revised version accepted 7 April 1997.
Corresponding author: Dr B. G. Corney.

Materials and methods

Bacterial strains

The bacterial strains (Table 1) were obtained from L. Smythe (WHO Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, Queensland, Australia) except for *L. borgpetersenii* serovar hardjobovis strains 11135 and Hb-15b-067, which were obtained from the Commonwealth Serum Laboratories (Parkville, Victoria, Australia) and Dr C. Bolin (National Animal Disease Center, Ames, Iowa, USA), respectively.

RAPD

DNA for RAPD was prepared by rapid lysis of pelleted cells [8]. RAPD was performed with primers L10 and US as described by Corney *et al.* [8], and primers L7 (5'TGCACCCTCCCCCCGGCCGA3'), L9 (5'TCGCG-

CTAGAGATGGCGGGG3′), L11 (5′GGCCGGGGGG-AGGGTGCAC3′) and L12 (5′AGGCGGCGCCAGC-AGCGCG3′).

Agarose gel electrophoresis

Up to 20 μl of RAPD products were loaded on to agarose 2% submarine gels by either 'conventional' or 'dry' gel loading methods and electrophoresed for 2 h at 100 V (Pharmacia GNA100 electrophoresis cell) or 150 V (Pharmacia GNA200 electrophoresis cell). Gels were stained with ethidium bromide and photographed. RAPD profiles were compared by eye.

For 'conventional' loading, gel loading buffer consisting of bromphenol blue 0.25% w/v, xylene cyanol 0.25% w/v and sucrose 40% w/v [13] was diluted 1 in 6 in RAPD reaction mixture. Gels were submerged in electrophoresis buffer (0.5 × TBE; 0.045 M Tris-borate and 0.001 M ethylenediamine tetraacetic acid disodium salt) [13] and the mixture was loaded through the buffer into the wells. For 'dry loading', horizontal gels were cast as normal and were placed in the electrophoresis cell. Electrophoresis buffer was added to the cell until it was almost lapping the upper gel surface. Each sample was loaded into the dry wells without gel loading buffer, and voltage was applied for 1–2 min. The gels were submerged in buffer and electrophoresis was continued for 2 h.

Results

Comparison of 'dry' loading and 'conventional' gel loading

Preliminary results suggested that 'dry' loading gives clearer profiles with sharper bands than 'conventional' gel loading. To demonstrate the difference, duplicate agarose 2% gels were loaded with the same volumes of two RAPD reactions and run under identical conditions. 'Dry' loading was used for one gel, and 'conventional' loading was used for the other. 'Dry' loading gave better definition of bands than did 'conventional' loading. Faint bands were clearer and close bands were better resolved. 'Dry' loading was adopted for all RAPD experiments.

Analysis of serovars with primers L10 and US

Type strains from 45 serovars (as listed by Faine [14]), and two serovar hardjobovis strains, representing 21 serogroups, were analysed by RAPD with primers L10 and US. Each RAPD was performed up to three times. Thirteen serovars were from serogroup Pyrogenes.

Generally, each strain had a unique RAPD profile with each primer. Profiles were reproducible, although there were minor variations in band intensity in some cases. Representative profiles are shown in Figs. 1 and 2. The RAPD profiles were compared by eye, and the strains were placed in RAPD similarity groups 1–8 as shown in Table 1. Both primers yielded the same groupings. Groups 1–7 correlated with *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilii*, *L. kirschneri* and *L. meyeri*, respectively, according to published genome species assignments [10, 11, 14]. Thirteen serovars from serogroup Pyrogenes were distributed among five RAPD groups. The reference strain of serovar hamptoni (also from serogroup Pyrogenes) bore no resemblance to any other serovar, and was the only member of RAPD group 8.

The type strains of serovars ballum and arborea were exceptions to the above. They had identical profiles with primer L10, but differed by one band with primer US. Similarly, serovars pomona and kennewicki had identical profiles with primers L10 and US; serovars icterohaemorrhagiae and copenhageni were identical with primer L10 and similar with primer US as were serovars zanoni and robinsoni; and serovars hardjobovis and balcanica were similar with both primers.

Resolution of closely related serovars

As RAPD with primers L10 and US failed to resolve certain serovars, and yielded very similar profiles for others, additional primers L7, L9, L11 and L12 were tested on a selection of these serovars, i.e., pomona, kennewicki, robinsoni, zanoni, balcanica, and genotypes A (strain 11135) and B (strain Hb-15b-067) of hardjobovis [15]. The latter two strains differ by one band with primer L10 and are identical with US [8]. Primers L9, L11 and L12 failed to distinguish between pomona and kennewicki and were discarded. Primer L7 differentiated between the five strains (Fig. 3).

Discussion

RAPD on crude cell lysates generated specific and reproducible fragment profiles on agarose gels which were characteristic of each leptospiral serovar. Serovars could be assigned to groups that matched each genome species of *Leptospira* on the basis of overall pattern similarities.

This procedure has several advantages over other similar methods. Rigorous DNA purification is not required. 'Dry' loading the agarose gels gives them sufficient resolution and sensitivity to remove the need for PAGE and autoradiography and the associated hazards. Pattern matching is done by eye, obviating the need for sophisticated computer-based analysis systems. Thus, the technique allows presumptive genome species assignments or isolate typing with minimal capital outlay and minimal exposure to dangerous chemicals.

The fragment profiles were reproducible despite the use of non-standardised crude lysates in the RAPD

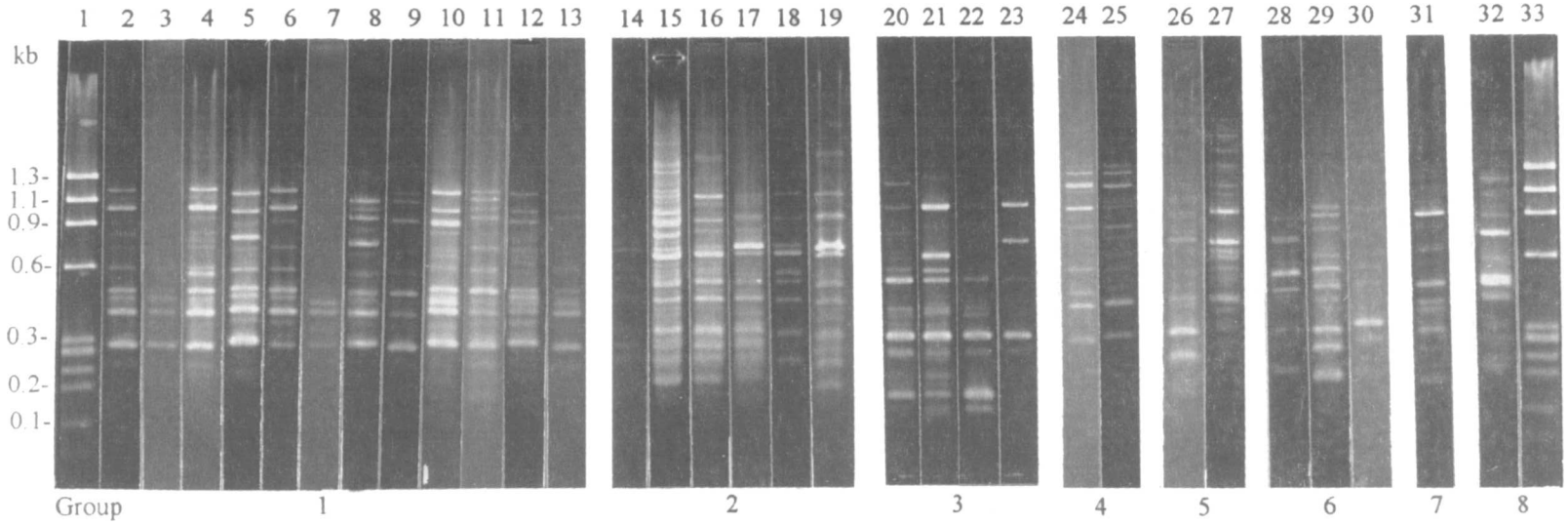

Fig. 1. RAPD profiles of representative serovars arranged into RAPD groups 1–8 following amplification with primer L10. Lanes **1** and **33**, ϕX174 replicative form DNA digested with *Hae*III; **2**, bindjei; **3**, bratislava; **4**, broomi; **5**, copenhageni; **6**, djasiman; **7**, guaratuba; **8**, icterohaemorrhagiae; **9**, kremastos; **10**, pomona; **11**, swajizak; **12**, valbuzzi; **13**, zanoni; **14**, arborea; **15**, balcanica; **16**, ballum; **17**, hardjobovis genotype A; **18**, javanica; **19**, kwale; **20**, alexi; **21**, princestown; **22**, shermani; **23**, varela; **24**, louisiana; **25**, myocastoris; **26**, celledoni; **27**, sarmin; **28**, bulgarica; **29**, cyanopteri; **30**, grippotyphosa; **31**, ranarum; **32**, hamptoni.

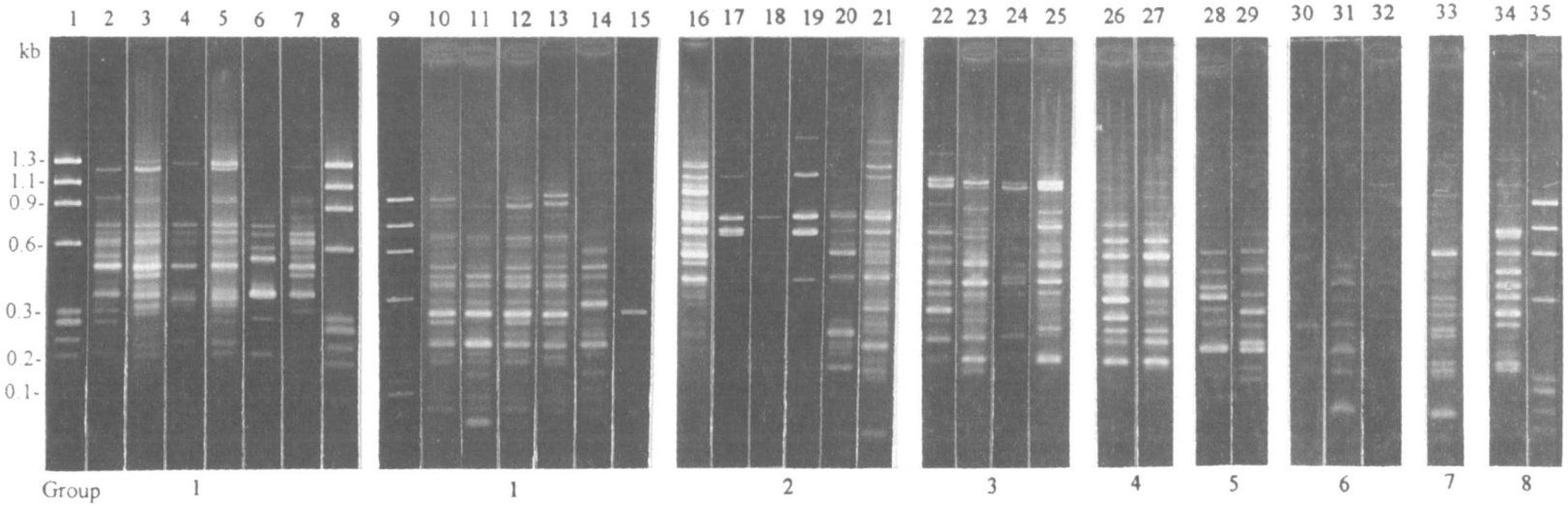

Fig. 2. RAPD profiles of representative serovars arranged into RAPD groups 1–8 following amplification with primer US. Lanes **1**, **8**, **9**, and **35**, ϕX174 replicative form DNA digested with *Hae*III; **2**, bratislava; **3**, copenhageni; **4**, guaratuba; **5**, pomona; **6**, swajizak; **7**, zanoni; **10**, bindjei; **11**, broomi; **12**, djasiman; **13**, icterohaemorrhagiae; **14**, kremastos; **15**, valbuzzi; **16**, arborea; **17**, balcanica; **18**, ballum; **19**, hardjobovis genotype A; **20**, javanica; **21**, kwale; **22**, alexi; **23**, princestown; **24**, shermani; **25**, varela; **26**, louisiana; **27**, myocastoris; **28**, celledoni; **29**, sarmin; **30**, bulgarica; **31**, cyanopteri; **32**, grippotyphosa; **33**, ranarum; **34**, hamptoni.

Table 1. Strains of *Leptospira* used in this study with their genome species and RAPD group assignments

Serovar	Strain	Serogroup	Genome species*	RAPD group
abramis	Abraham	Pyrogenes	*L. interrogans*	1
australis	Ballico	Australis	*L. interrogans*	1
autumnalis	Akiyami A	Autumnalis	*L. interrogans*	1
bataviae	Swart	Bataviae	*L. interrogans*	1
biggis	Biggs	Pyrogenes	*L. interrogans*	1
bindjei	Bindjei	Canicola	*L. interrogans*	1
bratislava	Jež-bratislava	Australis	*L. interrogans*	1
broomi	Patane	Canicola	*L. interrogans*	1
camlo	LT 64-67	Pyrogenes	*L. interrogans*	1
canicola	Hond Utrecht IV	Canicola	*L. interrogans*	1
copenhageni	M 20	Icterohaemorrhagiae	*L. interrogans*	1
djasiman	Djasiman	Djasiman	*L. interrogans*	1
guaratuba	An 7705	Pyrogenes	NA	1
hardjoprajitno	Hardjoprajitno	Sejroe	*L. interrogans*	1
hebdomadis	Hebdomadis	Hebdomadis	*L. interrogans*	1
icterohaemorrhagiae	RGA	Icterohaemorrhagiae	*L. interrogans*	1
kennewicki	LT 1026	Pomona	*L. interrogans*	1
kremastos	Kremastos	Hebdomadis	*L. interrogans*	1
mankarso	Mankarso	Icterohaemorrhagiae	*L. interrogans*	1
pomona	Pomona	Pomona	*L. interrogans*	1
pyrogenes	Salinem	Pyrogenes	*L. interrogans*	1
robinsoni	Robinson	Pyrogenes	*L. interrogans*	1
swajizak	Swajizak	Mini	NA	1
valbuzzi	Valbuzzi	Grippotyphosa	NA	1
zanoni	Zanoni	Pyrogenes	*L. interrogans*	1
arborea	Arborea	Ballum	NA	2
balcanica	1627 Burgass	Sejroe	*L. borgpetersenii*	2
ballum	Mus 127	Ballum	*L. borgpetersenii*	2
hardjobovis	11135 and Hb-15b-067	Sejroe	*L. borgpetersenii*	2
javanica	Bat 46	Javanica	*L. borgpetersenii*	2
kwale	Julu	Pyrogenes	*L. borgpetersenii*	2
sejroe	M 84	Sejroe	*L. borgpetersenii*	2
tarassovi	Perepelicin	Tarassovi	*L. borgpetersenii*	2
alexi	HS 616	Pyrogenes	*L. interrogans*	3
princestown	TRVL 112499	Pryogenes	*L. santarosai*	3
shermani	1342 K (= LT 821)	Shermani	*L. santarosai*	3
varela	1019	Pryogenes	*L. santarosai*	3
louisiana	LSU 1945	Louisiana	*L. noguchii*	4
myocastoris	LSU 1551	Pyrogenes	NA	4
celledoni	Celledoni	Celledoni	*L. weilii*	5
sarmin	Sarmin	Sarmin	*L. weilii*	5
bulgarica	Nikolaevo	Autumnalis	*L. kirschneri*	6
cyanopteri	3522 C	Cyanopteri	*L. kirschneri*	6
grippotyphosa	Moskva V	Grippotyphosa	*L. interrogans* or *L. kirschneri*	6
ranarum	ICF	Ranarum	*L. meyeri*	7
hamptoni	Hampton	Pyrogenes	NA	8

NA, not assigned.
*Genome species assignments are from Ramadass *et al.* [10] and Yasuda *et al.* [11] except for serovars alexi, broomi, camlo, kwale, mankarso, princestown, robinsoni and varela, which were taken from the serovar list published by Faine [14].

reactions. Preliminary experiments demonstrated that consistent profiles could be obtained over a wide range of DNA concentrations (up to 10 000-fold; results not shown). Similar results were reported by Welsh and McClelland [2], who attributed the reproducibility of their RAPD profiles to the use of a low annealing temperature in the first few amplification cycles followed by a high annealing temperature for the rest of the cycles.

This method has been used to identify bovine isolates of *Leptospira* [8]. The fact that almost all serovars examined in this study had unique RAPD profiles strongly suggests that a simplified RAPD such as this could be used for typing isolates from a wide variety of sources. It is possible to design an RAPD-procedure with a predetermined degree of resolution depending on the primer used. For example, primers such as L10 and US were useful for examining similarities between isolates and serovars, perhaps as a tool for making presumptive species assignments. Primer L7 provided finer resolution and was useful for differentiating between closely related serovars and genotypes.

The serovar groupings produced by simplified RAPD agreed with species assignments made by DNA hybridisation [10, 11] and those made by Ralph *et al.* [7] by AP-PCR and other molecular techniques. Serovar groupings by DNA hybridisation analysis [11] did not correspond with serogroups. Similarly, serogroup Pyrogenes reference strains were distributed among five RAPD groups, corresponding to four genome species. The fifth group contained only one member, serovar hamptoni, and did not correlate with any recognised genome species. Serovar hamptoni has

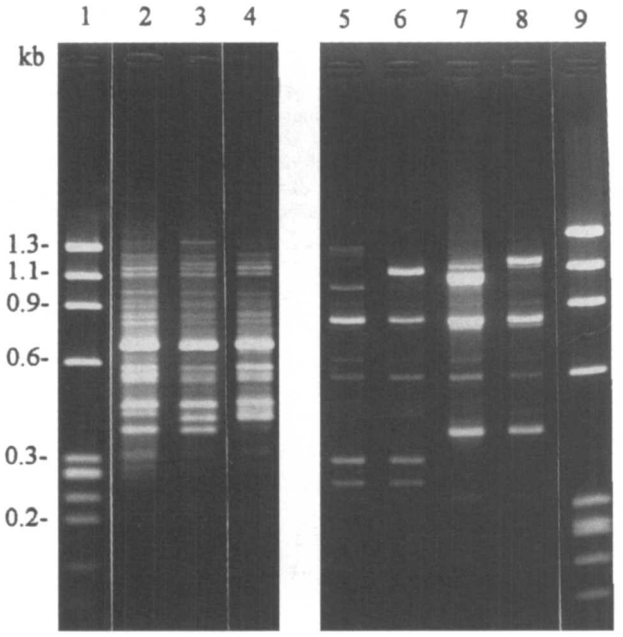

Fig. 3. RAPD profiles of hard-to-resolve serovars obtained by amplification with primer L7. Lanes **1** and **9**, ϕX174 replicative form DNA digested with *Hae*III; **2**, hardjobovis genotype A; **3**, hardjobovis genotype B; **4**, balcanica; **5**, robinsoni; **6**, zanoni; **7**, pomona; **8**, kennewicki.

not yet been assigned to a genome species. The results of the present study suggest that this serovar may represent an additional genome species. However, Pérolat *et al.* [16] found similarities in the ribotypes of hamptoni and serovars of *L. borgpetersenii*. DNA hybridisation comparisons of serovar hamptoni with other serovars are necessary to clarify its taxonomic position.

The only serovar for which the RAPD grouping did not correspond with prior genome species assignments was alexi. Faine [14] listed this serovar in *L. interrogans*, whereas this study placed it in RAPD group 3, which corresponds with *L. santarosai*. Similarly, Pérolat *et al.* [16] reported similarities between the ribotypes of serovar alexi and those of *L. santarosai*. This serovar was not included in any of the published DNA hybridisation studies [10, 11].

Serovars guaratuba, swajizak, valbuzzi, arborea and myocastoris are not at present assigned to a genome species. The first three were placed in RAPD group 1, arborea in RAPD group 2 and myocastoris in RAPD group 4 corresponding to *L. interrogans*, *L. borgpetersenii* and *L. noguchii*, respectively. Previously it was suggested that serovar swajizak be grouped with other *L. interrogans* serovars in a small scale survey of serovars that may be associated with cattle [8].

Ralph *et al.* [7] queried the validity of *L. weilii* as a single species because of the degree of difference they observed between serovars celledoni and sarmin in AP-PCR and mapped restriction site polymorphism (MRSP) analysis. In the RAPD analyses, reference strains of these serovars were sufficiently similar to be placed in the same group corresponding to *L. weilii*. This disparity in results probably reflects differences in resolution between primers, similar to differences observed in the performance of L10 and US when compared with L7.

Other workers disagree on the placement of serovar grippotyphosa. Yasuda *et al.* [11] assigned grippotyphosa to *L. interrogans*, whereas Ramadass *et al.* [10] assigned the serovar to their newly proposed species *L. kirschneri*. The RAPD results in the present study and the AP-PCR and MRSP results of Ralph *et al.* [7] support the assignment of serovar grippotyphosa to *L. kirschneri*.

In conclusion, RAPD coupled with agarose gel electrophoresis is a rapid and simple method for making preliminary genome species assignments. RAPD also has potential as a rapid method for serovar identification.

We thank L. Smythe for critically appraising the manuscript and the staff of the WHO Collaborating Centre for Reference and Research on Leptospirosis, Carole Bolin, and Commonwealth Serum Laboratories for supplying the cultures.

References

1. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 1990; **18**: 6531–6535.
2. Welsh J, McClelland M. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* 1990; **18**: 7213–7218.
3. Brousseau R, Saint-Onge A, Préfontaine G, Masson L, Cabana J. Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. *Appl Environ Microbiol* 1993; **59**: 114–119.
4. Jayarao BM, Bassam BJ, Caetano-Anollés G, Gresshoff PM, Oliver SP. Subtyping of *Streptococcus uberis* by DNA amplification fingerprinting. *J Clin Microbiol* 1992; **30**: 1347–1350.
5. Mazurier S, van de Giessen A, Heuvelman K, Wernars K. RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. *Lett Appl Microbiol* 1992; **14**: 260–262.
6. Welsh J, Pretzman C, Postic D, Girons IS, Baranton G, McClelland M. Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* into three distinct phyletic groups. *Int J Syst Bacteriol* 1992; **42**: 370–377.
7. 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.
8. 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.
9. Pérolat P, Merien F, Ellis WA, Baranton G. Characterization of *Leptospira* isolates from serovar hardjo by ribotyping, arbitrarily primed PCR, and mapped restriction site polymorphisms. *J Clin Microbiol* 1994; **32**: 1949–1957.
10. 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.
11. 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.

12. Ramadass P, Jarvis BDW, Corner RJ, Cinco M, Marshall RB. DNA relatedness among strains of *Leptospira biflexa*. *Int J Syst Bacteriol* 1990; **40**: 231–235.
13. Sambrook J, Fritsch EF, Maniatis T (eds). Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press. 1989.
14. Faine S. *Leptospira* and leptospirosis. Boca Raton, FL, CRC Press. 1994.
15. 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.
16. Pérolat P, Lecuyer I, Postic D, Baranton G. Diversity of ribosomal DNA fingerprints of *Leptospira* serovars provides a database for subtyping and species assignation. *Res Microbiol* 1993; **144**: 5–15.