ARTÍCULO ORIGINAL

ISSN 0325-7541
Revista Argentina de Microbiología (2011) 43: 251-255

Multiple-locus variable-number tandem repeat analysis of reference strains used for the diagnosis of leptospirosis in Argentina

MARÍA E. PAVAN[1]*, BIBIANA BRIHUEGA[2, 5], MARÍA J. PETTINARI[3], FABIAN CAIRÓ[1, 4]

[1]Biochemiq S.A., Laboratorio de Biología Molecular, Ciudad Autónoma de Buenos Aires;
[2]World Organisation for Animal Health (OIE) Leptospirosis Reference Laboratory, Instituto de Patobiología, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas (CICVyA), Instituto Nacional de Tecnología Agropecuaria (INTA), Hurlingham, Buenos Aires; [3]Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, and [4]Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires; [5]Facultad de Ciencias Veterinarias, Universidad del Salvador, Pilar, Buenos Aires, Argentina.
**Correspondence. E-mail: mpavan@biochemiq.com*

ABSTRACT

Leptospirosis is a worldwide zoonosis caused by a spirochete that belongs to the genus *Leptospira.* In the last years, new methods, such as the PCR-based multiple-locus variable-number tandem repeat analysis (MLVA), have been developed for the genotyping of leptospires. In the present work, the MLVA patterns for all reference strains used in Argentina for bovine, ovine, porcine, equine, caprine and canine leptospirosis diagnosis, as well as in human and wild animal diagnosis, were obtained. MLVA results are presented in such a way that they can be readily used for the identification of these strains by the simple and direct comparison of agarose gels. Making the use and interpretation of the MLVA for leptospires typing easier will help increase the use of this method as a routine procedure for human and animal diagnosis, for epidemiological studies, vaccine control and other applications.

Key words: *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira borgpetersenii*, reference strains, MLVA, VNTR, genotyping

RESUMEN

***Multiple-locus variable-number tandem repeat analysis* de cepas de referencia usadas para el diagnóstico de leptospirosis en Argentina.** La leptospirosis es una zoonosis de distribución global causada por una espiroqueta perteneciente al género *Leptospira.* En los últimos años se han desarrollado nuevos métodos para la genotipificación de las leptospiras, entre ellos el denominado *multiple-locus variable-number tandem repeat analysis* (MLVA). En este trabajo se obtuvieron los patrones de MLVA de todas las cepas de referencia utilizadas en la Argentina para el diagnóstico de leptospirosis en bovinos, ovinos, porcinos, equinos, caprinos y perros, y que también son utilizadas en el diagnóstico de leptospirosis en humanos y en animales salvajes. Los resultados del MLVA se muestran de manera tal que pueden ser fácilmente utilizados para la identificación de estas cepas por simple comparación visual de geles de agarosa. Al facilitar el uso y la interpretación del MLVA para la tipificación de leptospiras, se ayudará a difundir la utilización rutinaria de este método en el diagnóstico humano y animal, en estudios epidemiológicos y para el control de vacunas, entre otras aplicaciones.

Palabras clave: *Leptospira interrogans*, *Leptospira kirschneri, Leptospira borgpetersenii*, cepas de referencia, MLVA, VNTR, genotipificación

INTRODUCTION

Leptospirosis is a worldwide zoonosis caused by a spirochete that belongs to the genus *Leptospira.* The first classifications of pathogenic leptospires were serological, establishing serogroups and serovars through antigenic relationships between strains. Later, the genus *Leptospira* was divided into 20 species considering DNA relatednesses (2, 5, 7, 9, 13, 14, 17, 18, 21). Nowadays, serological and molecular classifications coexist, and due to the lack of correspondence between them, new *Leptospira* strains should be characterized by both molecular and serological approaches (1, 6).

In Argentina, the reference method for serological diagnosis of leptospirosis is the microscopic agglutination test (MAT), which requires the maintenance of a collection of reference strains and the corresponding antisera (4). After many years of experience, the Argentine Permanent Scientific Commission for Leptospirosis has recommended a group of antigenic strains including *Leptospira interrogans, Leptospira borgpetersenii* and *Leptospira kirschneri* strains to be used for MAT in leptospirosis in

cows, sheep, pigs, horses, goats and dogs (4). These strains are also used for MAT in human and wild animal leptospirosis, along with others that are used specifically in these cases.

In the last years, new advances in the genotyping of leptospires have been made. Majed *et al.* (8) used an innovative PCR-based typing scheme, the multiple-locus variable-number tandem repeat analysis (MLVA), for the molecular typing of *L. interrogans*. The authors examined a worldwide reference collection using variable-number tandem repeat (VNTR) loci, and defined 47 unique genotypes (8). Later, Slack *et al.* (16, 19) developed a different MLVA to examine *L. interrogans* reference strains, and clinical isolates from Australia. MLVA was successfully used for the identification of strains, even within the same serovar (11, 12, 16, 19). Salaün *et al.* (15) improved the previously described MLVA (8) to allow the typing not only of *L. interrogans* strains but also of strains belonging to two other pathogenic species, *L. kirschneri* and *L. borgpetersenii.*

MLVA involves the analysis of multiple loci of VNTR, repeated DNA sequences of varying copy numbers that are generated by slipped strand mispairing during DNA replication (12, 20). The method consists in amplifying these variable length regions showing polymorphism to differentiate the repeat copy number through the size of the resultant amplicon. This technique is simple and easy to implement in a laboratory without sophisticated equipment. In this way, MLVA is useful for the proper identification of *Leptospira* strains and is ideal for epidemiological studies. However, the results obtained can be difficult to interpret using published data, because only raw copy numbers are usually reported. Therefore, calculations involving the size of each repeat, the flanking regions and the different possible numbers of repeats must be made in order to correctly interpret the molecular weights of the DNA fragments that result from the amplifications.

The aim of this work was to present the MLVA genotyping of reference strains used in Argentina for the diagnosis of leptospirosis, in such a way that the MLVA patterns can be readily used for the identification of these strains by the simple and direct comparison of agarose gel electrophoresis results.

MATERIALS AND METHODS

The *L. interrogans*, *L. borgpetersenii* and *L. kirschneri* reference strains used in this study, including all strains recommended by the Argentine Permanent Scientific Commission for Leptospirosis, are shown in Table 1. Each strain was grown in EMJH medium (Difco Laboratories) for at least 5 days at 28 °C. Culture samples (100 µl) were incubated at 100 °C for 10 min and used directly as a DNA template in MLVA strain typing procedures. The primers described by Majed *et al.* (8) flanking the VNTR4, VNTR7, VNTR9, VNTR10, VNTR19, VNTR23 and VNTR31 loci were used for *L. interrogans* whereas the oligonucleotides described by Salaün *et al.* (15) were used for *L. borgpetersenii* and *L. kirschneri*, including those flanking the new VNTR-Lb4 and VNTR-Lb5 loci, and the previously found VNTR4, VNTR7 and VNTR10 loci [herein named VNTR-4bis, VNTR-7bis and VNTR-10bis in order to differentiate PCRs from those performed with the primers developed by Majed *et al.* (8)]. Each reaction mixture contained a PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), 200 µM of each dNTP, 2 mM $MgCl_2$, 1.25 U of Taq DNA polymerase (Invitrogen), 2 µM each corresponding primer, and 5 µl of DNA template in a final volume of 50 µl. PCRs were carried out in a MiniCycler PTC-150 (MJ Research) as follows: 94 °C for 5 min, followed by 35 cycles of denaturalization at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s, with a final cycle at 72 °C during 10 min. Each amplified sample (14 µl) was subjected to electrophoresis in a 2.2 % agarose gel in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) with 0.2 µg/ml ethidium bromide at 100 V for 50 min. Amplified DNA bands were visualized upon UV light exposure (DynaLight, Labnet). Amplicon sizes were estimated using CienMarker (Biodynamics).

RESULTS AND DISCUSSION

The reference strains analyzed in the present work belong to *L. interrogans, L. borgpetersenii* and *L. kirschneri,*

Table 1. Reference strains used in Argentina for the diagnosis of leptospirosis for domestic animals analysed in this work

Species	Serogroup	Serovar	Strain	Animal species[(1)]	Strain procedence[(2)]
L. interrogans	Canicola	Canicola	Hond Utrecht IV	Hu, Do, Co, Sh, Pi, Ho, Go, Wi	I
	Icterohaemorrhagiae	Copenhageni	M20	Hu, Do, Co, Sh, Pi, Ho, Go, Wi	I
	Pomona	Pomona	Pomona	Hu, Do, Co, Sh, Pi, Ho, Go, Wi	I
	Pyrogenes	Pyrogenes	Salinem	Hu, Do, Wi	I, II, III
	Sejroe	Wolffi	3705	Hu, Co, Sh, Pi, Ho, Go, Wi	I, II, III
		Hardjo	Hardjoprajitno[(3)]	Hu, Co, Sh, Pi, Ho, Go, Wi	I, II, III
L. borgpetersenii	Ballum	Castellonis	Castellon 3	Hu, Do, Co, Sh, Pi, Ho, Go, Wi	I, II, III
	Tarassovi	Tarassovi	Perepelicin	Hu, Co, Sh, Pi, Ho, Go, Wi	I, II, III
L. kirschneri	Grippotyphosa	Grippotyphosa	Moskva V	Hu, Co, Sh, Pi, Ho, Go, Wi	I, II, III, IV

[(1)] According to the animal species from which the serum to be analyzed is obtained, the Argentine Permanent Scientific Commission for Leptospirosis recommended which *Leptospira* strains should be used for MAT diagnosis. Abbreviations for each animal species: Hu, humans; Do, dogs; Co, cows; Sh, sheep; Pi, pigs; Ho, horses; Go, goats; Wi, wild animals. [(2)]Strain source: I, World Organisation for Animal Health (OIE) Leptospirosis Reference Laboratory, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas (CICVyA), Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina; II, Institut Pasteur, France; III, Instituto Fiocruz, San Salvador, Brazil; IV, Pan American Health Organization, through the División Laboratorios Veterinarios (DILAVE), Uruguay. [(3)]Strain only recommended by INTA-OIE Leptospirosis Reference Laboratory.

therefore, we used two different oligonucleotide sets (8, 15) according to the corresponding species, which allowed the correct genotyping of all reference strains.

The MLVA genotypes of *L. interrogans* reference strains Hond Utrecht IV, M20, Pomona, Salinem, 3705 and Hardjoprajitno, recommended by the Argentine Permanent Scientific Commission for Leptospirosis and INTA-OIE Leptopirosis Reference Laboratory to perform MAT in human, domestic and wild animal leptospirosis, were obtained by using seven of the loci described by Majed *et al*. (8). The patterns obtained for these strains are shown in Figure 1. The MLVA profiles of the six reference strains are absolutely different, and therefore, they can be readily recognized. Strains Hond Utrecht IV (serovar Canicola), M20 (serovar Copenhageni) and Pomona (serovar Pomona) yielded similar results to those reported by Majed *et al.* (8). Strain Pomona has an easily identifiable pattern (Figure 1C) that can be differentiated not only from that of other reference strains, but also from all *L. interrogans* MLVA genotypes described in Argentina and Brazil, corresponding to strains with genotypes A, B, C and D from serovar Pomona (10, 11, 12). The profile obtained for strain M20 (Figure 1B) could be rapidly singled out from that of the other five reference *L. interrogans* strains. However, when using MLVA for typing unknown isolates, it must be taken into account that the pattern of strain M20 is identical to that of two strains from serovar Icterohaemorrhagiae: Ictero No. 1 and RGA, used for human diagnosis (12).

When the MLVA patterns obtained for strains Salinem (serovar Pyrogenes), 3705 (serovar Wolffi) and Hardjoprajitno (serovar Hardjo) were compared with previously published results for these strains (8), discrepancies in some amplification fragment molecular weights were observed. Some of the small discrepancies with previous results could be attributed to inevitable electrophoretic run variations, but differences of nearly a hundred base pairs were observed compared to published data (8) for the VNTR9 Hardjoprajitno amplicon and the large VNTR19 Salinem amplicon, implicating a difference of two repeats (approximately 45 bp each) for the loci considered. In order to verify that the strains tested had not been mislabeled,

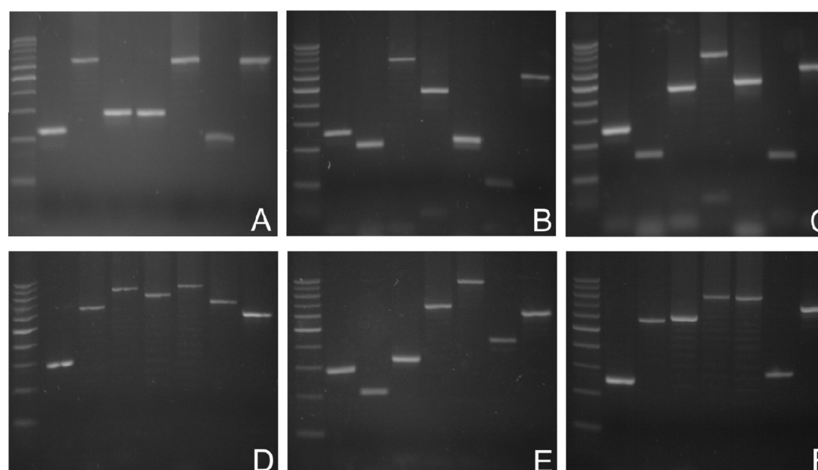

Figure 1. MLVA patterns for the following *L. interrogans* strains: A, Hond Utrecht IV (serovar Canicola); B, M20 (serovar Copenhageni); C, Pomona (serovar Pomona); D, Salinem (serovar Pyrogenes); E, strain 3705 (serovar Wolffi); F, strain Hardjoprajitno (serovar Hardjo). The VNTR loci evaluated were: VNTR4 (lanes 2), VNTR7 (lanes 3), VNTR9 (lanes 4), VNTR10 (lanes 5), VNTR19 (lanes 6), VNTR23 (lanes 7) and VNTR31 (lanes 8). Lanes 1: CienMarker.

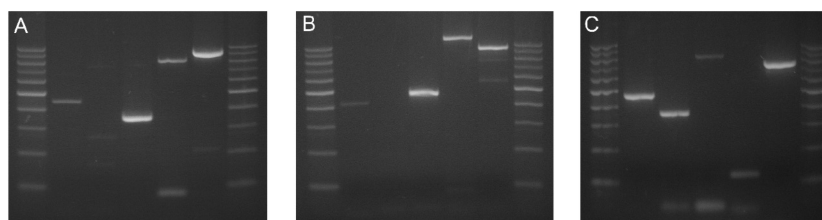

Figure 2. MLVA patterns for the following *Leptospira* strains: A, Castellon 3 from *L. borgpetersenii;* B, Perepelicin from *L. borgpetersenii*; C, Moskva V from *L. kirschneri*. The VNTR loci evaluated were: VNTR-4bis (lanes 2), VNTR-7bis (lanes 3), VNTR-10bis (lanes 4), VNTR-Lb4 (lanes 5), and VNTR-Lb5 (lanes 6). Lanes 1 and 7: CienMarker.

samples of the same strains were obtained from two additional culture collections, those from the Institut Pasteur, France, and from Fiocruz, Brazil. Results obtained with the new samples were identical to those observed for the first samples, consequently, the patterns for Salinem, 3705 and Hardjoprajitno strains can be reliably considered to be the ones shown in this work.

The scheme proposed for *L. interrogans* (8) cannot be applied to strains belonging to *L. borgpetersenii* and *L. kirschneri* species because some of the loci give faint bands, multiple bands or do not amplify at all (15). For this reason, MLVA patterns for the Argentine reference strains Moskva V from *L. kirschneri,* and Castellon 3 and Perepelicin from *L. borgpetersenii* were obtained using the loci proposed by Salaün *et al.* (15) (Figure 2). The typing of Moskva V strain, serovar Grippotyphosa, was problematic. Several attempts to amplify the VNTR loci of this strain from the INTA and Institut Pasteur culture collections were unsuccessful. The Moskva V strain from Fiocruz could be genotyped, but the MLVA pattern obtained (Figure 2C) differed from the previously published one (15). In an effort to clarify this situation, this strain was also obtained from the Pan American Health Organization. This last strain gave unexpected results, including high molecular weight bands, duplicate bands or no amplicons for different loci.

Strain Castellon 3, serovar Castellonis, yielded MLVA patterns comparable to those previously reported (15), but a difference was observed for the band expected for VNTR-4bis according to the source of the strain used, ranging from readily visible (strain from Fiocruz, shown in Figure 2A), tenuous (strain from INTA) or missing (strain from Institut Pasteur). Strain Perepelicin, serovar Tarassovi, from the INTA culture collection gave a similar pattern (Figure 2B) to the previously reported one (15), but differed in the size of the VNTR-10bis amplification band, which was a little larger than expected. However, when this strain obtained from different sources (Fiocruz and Institut Pasteur) was tested, an identical pattern was obtained, showing the same discrepancy with respect to the published pattern. In agreement with Salaün e*t al.*'s findings (15), no amplification band was obtained for locus VNTR-4bis, except for a very weak band that was observed for the Fiocruz strain, as shown in Figure 2B. The absence of an amplicon for the VNTR-7bis locus indicates that Castellon 3 and Perepelicin strains belong to the species *L. borgpetersenii* (Figure 2A and 2B).

The reference strains used for obtaining antibodies for MAT or for vaccine production, as well as those used in vaccine control challenging assays must be periodically characterized in order to avoid important problems due to mismanagement or contamination. Mislabeling during subculturing or strain switching are ever-present risks as it could be demonstrated during a recent monitoring of two Brazilian *Leptospira* strain collections (3). Reference strains are also needed for epidemiological studies, and each country should maintain and control its own collection with the best tools available, due to the current difficulties to acquire pathogenic microorganisms.

MLVA is a simple, rapid and economical method, but the interpretation of the results obtained, including the comparison with reference strains, is difficult because it has to be made based on VNTR copy number variations. The aim of this work was to facilitate the use and interpretation of this technique for leptospire typing, presenting the MLVA patterns for reference strains used in Argentina in such a way that they can be differentiated by simple visual comparison of agarose electrophoresis results. This will allow MLVA to be incorporated as a routine procedure in strain collection monitoring, epidemiological studies, typing of leptospires isolated from humans and animals, vaccine control and other uses. The widespread application of MLVA will increase the reliability of leptospire typing complementing the traditional serological techniques.

REFERENCES

1. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM; Peru-United States Leptospirosis Consortium. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 2003; 3: 757-71.
2. Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC, Weyant RS. Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. Int J Syst Bacteriol 1999; 49: 839-58.
3. Cerqueira GM, McBride AJA, Queiroz A, Pinto LS, Silva EF, Hartskeerl RA, Reis MG, Ko AL, Dellagostin OA. Monitoring *Leptospira* strain collections: the need for quality control. Am J Trop Med Hyg 2010; 82: 83-7.
4. Comisión Científica Permanente sobre Leptospirosis. Manual de leptospirosis. Asociación Argentina de Veterinarios de Laboratorios de Diagnóstico, 1994.
5. International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of *Leptospiraceae*. Minutes of the closed meeting, 18 September 2007, Quito, Ecuador. Int J Syst Evol Microbiol 2008; 58: 1049-50.
6. Levett PN. Leptospirosis. Clin Microbiol Rev 2001; 4: 296-326.
7. Levett PN, Morey RE, Galloway RL, Steigerwalt AG. *Leptospira broomii* sp. nov., isolated from humans with leptospirosis. Int J Syst Evol Microbiol 2006; 56: 671-3.
8. Majed Z, Bellenger E, Postic D, Pourcel C, Baranton G, Picardeau M. Identification of variable-number tandem-repeat loci in *Leptospira interrogans* sensu stricto. J Clin Microbiol 2005; 43: 539-45.
9. Matthias MA, Ricaldi JN, Cespedes M, Díaz MM, Galloway RL, Saito M, Steigerwalt AG, Patra KP, Ore CV, Gotuzzo E, Gilman RH, Levett PN, Vinetz JM. Human leptospirosis caused by a new, antigenically unique *Leptospira* associated with a *Rattus* species reservoir in the Peruvian Amazon. PLoS Negl Trop Dis 2008; 2: e213.
10. Miraglia F, Moreno AM, Ribeiro Gomes C, Paixão R, Liuson E, Morais ZM, Maiorka P, Kömmling Seixas F, Dellagostin OA, Arruda Vasconcellos S. Isolation and characterization of *Leptospira interrogans* from pigs slaughtered in São Paulo state, Brazil. Braz J Microbiol 2008; 39: 501-7.
11. Pavan ME, Cairó F, Brihuega B, Samartino L. Multiple-locus variable-number tandem repeat analysis (MLVA) of *Leptospira interrogans* serovar Pomona from Argentina reveals four

new genotypes. Comp Immunol Microbiol Infect Dis 2008; 31: 37-45.

12. Pavan ME, Cairó F, Pettinari MJ, Samartino L, Brihuega B. Genotyping of *Leptospira interrogans* strains from Argentina by multiple-locus variable-number tandem repeat Analysis (MLVA). Comp Immunol Microbiol Infect Dis 2011; 34: 135-41.
13. Perolat P, Chappel RJ, Adler B, Baranton G, Bulach DM, Billinghurst ML, Letocart M, Merien F, Serrano MS. *Leptospira fainei* sp. nov., isolated from pigs in Australia. Int J Syst Bacteriol 1998; 48: 851-8.
14. Ramadass P, Jarvis BD, Corner RJ, Penny D, Marshall RB. Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. Int J Syst Bacteriol 1992; 42: 215-9.
15. Salaün L, Mérien F, Gurianova S, Baranton G, Picardeau M. Application of multilocus variable-number tandem-repeat analysis for molecular typing of the agent of leptospirosis. J Clin Microbiol 2006; 44: 3954-62.
16. Slack AT, Dohnt MF, Symonds ML, Smythe LD. Development of a multiple-locus variable number of tandem repeat analysis (MLVA) *for Leptospira interrrogans* and its application to *Leptospira interrogans* serovar Australis isolates from Far North Queensland, Australia. Ann Clin Microbiol Antimicrob 2005; 4: 10.
17. Slack AT, Kalambaheti T, Symonds ML, Dohnt MF, Galloway RL, Steigerwalt AG, Chaicumpa W, Bunyaraksyotin G, Craig S, Harrower BJ, Smythe LD. *Leptospira wolffii* sp. nov., isolated from a human with suspected leptospirosis in Thailand. Int J Syst Evol Microbiol 2008; 58: 2305-8.
18. Slack AT, Khairani-Bejo S, Symonds ML, Dohnt MF, Galloway RL, Steigerwalt AG, Bahaman AR, Craig S, Harrower BJ, Smythe LD. *Leptospira kmetyi* sp. nov., isolated from an environmental source in Malaysia. Int J Syst Evol Microbiol 2009; 59: 705-8.
19. Slack A, Symonds M, Dohnt M, Smythe L. An improved multiple-locus variable number of tandem repeats analysis for *Leptospira interrogans* serovar Australis: a comparison with fluorescent amplified fragment length polymorphism analysis and its use to redefine the molecular epidemiology of this serovar in Queensland, Australia. J Med Microbiol 2006; 55: 1549-57.
20. van Belkum A, Scherer S, van Alphen L, Verbrugh H. Short-sequence DNA repeats in prokaryotic genomes. Microbiol Mol Biol Rev 1998; 62: 275-93.
21. 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: 407-15.

Recibido: 15/03/2011 – Aceptado: 12/08/2011