ISOLATION AND MOLECULAR CHARACTERIZATION OF *Leptospira borgpetersenii* SEROVAR Ballum

[AISLAMIENTO Y CARACTERIZACIÓN MOLECULAR DE *Leptospira borgpetersenii* SEROVARIEDAD Ballum]

Carlos Alfredo Carmona Gasca[1], Albert I Ko[2], Niyaz Ahmed[3], Alejandro De la Peña-Moctezuma[1]

[1]Grupo de Investigación en Leptospira y Leptospirosis, Centro de Enseñanza Investigación y Extensión en Producción Animal en Altiplano, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México;
[2]Weill Cornell Medical College and Oswaldo Cruz Foundation / Brazilian Ministry of Health, Salvador, Brazil;
[3]Pathogen Biology Laboratory School of Life Sciences, University of Hyderabad, Room No. LS-296, School of Life Sciences, Central University P.O. Gachibowli, Hyderabad 500046, India
Email: delapema@unam.mx

SUMMARY

Rodents are the main reservoirs of pathogenic leptospires, spreading the organism to the environment and so the major risk factor for both, animals and humans to acquire leptospirosis. To assess such a role, 50 mice (*Mus musculus*) were caught in a dairy farm in the municipality of Teoloyucan, State of Mexico in central Mexico. Anti-*Leptospira* antibody titers (≥1:20), were obtained in 46 % (23) of the animals by the microscopic agglutination test (MAT), where the most common serovars detected were: Ballum 38 % (19), Canicola 10 % (5) and Australis 2 % (1). Three *Leptospira* isolates (6 %) were obtained by culture of mice kidneys macerates. The cultures were identified as *L. borgpetersenii* serovar Ballum by cross-MAT, IS*1533*-based PCR assay, *rrs2* (rRNA) sequencing, restriction fragment length polymorphism (RFLP) and by multiple locus sequencing typing (MLST). As far as we know, this is the first report on the isolation of *L. borgpetersenii* serovar Ballum recovered from *Mus musculus* in Mexico.

Keywords: *Mus musculus*; mouse; leptospirosis**.**

RESUMEN

Los roedores son el principal reservorio de leptospiras patógenas y por lo tanto diseminan al organismo en el ambiente, constituyendo un importante factor de riesgo para adquirir leptospirosis tanto para animales como para el hombre. Para evaluar tal papel, 50 ratones fueron capturados en una unidad de producción lechera en el municipio de Teoloyucan, Estado de México. Según el análisis morfométrico, todos los roedores fueron identificados como *Mus musculus*. Los títulos de anticuerpos séricos anti-*Leptospira* (≥1:20), se obtuvieron en el 46 % (23) de los ratones por la prueba de aglutinación microscópica (AM), donde los sueros reaccionaron con las serovariedades: Ballum 38 % (19), Canicola 10 % (5) y Australis 2 % (1). Tres aislados de *Leptospira* (6 %) fueron obtenidos por cultivo de macerados de riñones. Los cultivos fueron identificados como *L. borgpetersenii* serovariedad Ballum por aglutinación microscópica cruzada, PCR basado en amplificación de IS*1533*, secuenciación del gen *rrs2*, polimorfismo de la longitud de los fragmentos de restricción (RFLP) y por tipificación por secuencias de *locus* múltiples (MLST).

Palabras Clave: *Mus musculus*; ratón; leptospirosis.

INTRODUCTION

Leptospirosis is an infectious disease caused by pathogenic *Leptospira* serovars. Within this genus, thirteen pathogenic species have been identified: *L. alexanderi, L. alstonii, L. borgpetersenii, L. fainei, L. inadai, L. interrogans, L. kirschneri, L. licerasiae, L. noguchii, L. santarosai, L. terpstrae, L. weilii, L. wolffii* (International Committee on Systematics of Prokaryotes, 2008) and six non-pathogenic species: *L. biflexa, L. kmetyi, L. meyeri, L. vanthielii, L. wolbachii* and *L. yanagawae*, (Cerqueira and Picardeau, 2009; Adler and de la Peña-Moctezuma, 2010). These species include over 300 serovars, morphologically non distinguishable. *L. interrogans* includes the highest number of pathogenic serovars,

although other serovars from different species have also shown to cause disease in man and domestic animals (Ko *et al*., 2009).

Since the discovery of the disease, rodents have been recognized as the major reservoirs of pathogenic leptospires eliminating large numbers of infecting organism in the urine (Ko *et al*., 2009), thus participating directly and indirectly in the maintenance and transmission of leptospirosis to man and other animal species, coexisting in a geographical area in countries with favorable climatic conditions (Laras *et al.,* 2002). Despite improvement in diagnostic techniques and control measures, leptospirosis is still a major public health problem, mainly in under-developed areas of developing countries (Ko *et al.,* 2009).

Routine diagnosis of the disease is done by serological methods, microscopic agglutination test (MAT) and ELISA however; it is of high importance to recover the causing organism in culture, not just as an irrefutable proof of the disease, but also because the epidemiological data obtained to establish further prevention and control measures. The identification of the infecting strain provides information about the common serovars circulating in a given area. Isolates identification has been achieved among other techniques, by cross absorption agglutination (Ko *et al.,* 2009), restriction fragment length polymorphism, arbitrarily primed PCR (Perolat *et al.,* 1994), pulsed field gel electrophoresis (Herrmann *et al.,* 1992), fluorescent amplified fragment length polymorphism (Vijayachari *et al.,* 2004), analysis of the Variable Number of Tandem Repeats (VNTR) (Majed *et al.,* 2005), and the Multiple Locus Sequencing Typing (MLST) (Ahmed *et al.,* 2006). In Mexico, previously reported *Leptospira* isolates have been identified as *L. interrogans* by traditional serological approaches (Moles *et al.,* 2002). On the other hand, MAT studies have detected the presence of antibodies against several serovars, which nevertheless have not been yet isolated in Mexico. We report here three *Leptospira* isolates obtained from mice captured in a dairy farm in central Mexico and their characterization as *Leptospira borgpetersenii* serovar Ballum by cross microscopic agglutination, a *gspD – gspE* RFLP, the IS*1533*- based PCR assay, *rrs2* gene sequence analysis and ultimately by MLST.

MATERIALS AND METHODS

Rodents handling and bacteriological analysis

50 mice (*Mus musculus*), were captured from October to December 2007 in different areas of a dairy farm located in the municipality of Teoloyucan, State of Mexico in central Mexico (19º 44' 11" N, 99º 9' 1" E; 2,295 MASL). Fifty Sherman-type traps were used to capture mice and were randomly distributed leaving 10 meters between each trap to give the same effort unit per trap, during 12 hrs for 8 days, giving a total of 4,800 hrs / trap. The captured rodents were transported alive to the laboratory where they were identified by morphometric analysis and were euthanized by an intraperitoneal high dose of sodium penthobarbital (100-180 mg/kg), accordingly to Mexican regulations (NOM-062-ZOO-1999). Necropsies were practiced under aseptic conditions; blood was obtained by cardiac puncture and sera separated to perform the MAT. The liver and one kidney were inoculated in semisolid Fletcher and liquid EMJH media for culture of leptospires (Myers, 1985). 10^{-1}, 10^{-2} and 10^{-3} dilutions in each media were done and the cultures incubated at 30 °C for up to four months with periodical observations under dark field microscopy, until growth of leptospires was observed. Isolates were purified by dilutions up to 10^{-14}, considering the higher dilution showing growth as a pure isolate of *Leptospira.* Finally, specific polyclonal antiserum was prepared by weekly intravenous inoculation of pure cultures of the *Leptospira* isolates, during one month into two rabbits each isolate.

Cross-agglutination

Hyperimmune sera were produced in 1.5 kg rabbits by intravenous inoculation at one week intervals of 1, 2, 4 and 6 ml, recovered from the so called Dinger zone of the *Leptospira* isolates grown in Fletcher medium incubated at 30 °C per 7–10 days. Leptospires were inactivated at 56 °C for half an hour, before being inoculated into rabbits (Myers, 1985). Rabbit sera were clarified by centrifugation at 903 x*g* for 5 min and kept at -20 ºC until the MAT was performed. 29 *Leptospira* reference strains were used; the strains were kindly donated by the WHO/FAO/OIE-Collaborating Centre for Reference and Research on Leptospirosis, Australia and Western Pacific Region, Brisbane, Queensland, Australia (Table 1). The strains were cultured in liquid EMJH medium at 30 °C for 7 days and the MAT was performed as previously described (Myers, 1985). A titer of 1:20 was considered as the positive cutoff point (Sotomayor-Bonilla *et al.,* 2009).

DNA extraction and Polymerase Chain Reaction (PCR)

Isolates obtained were grown in 100 ml of EMJH medium and centrifuged at 10,000 x*g* for 30 min, DNA extraction was performed by the guanidine thiocyanate and chloroform-isoamyl alcohol method as described previously by Boom *et al.* (1990).

Table 1. Reference strains used for the MAT in this study.

Specie	Serogroup	Serovar	Strain
L. biflexa	Semaranga	Patoc	Patoc I
L. borgpetersenii	Ballum	Ballum	Mus-127
	Ballum	Castellonis	Castellon-3
	Sejroe	Hardjo	Hardjobovis
	Javanica	Javanica	Veldrat Bat 46
	Mini	Mini	Sari
	Sejroe	Sejroe	Sejroe M-84
	Tarassovi	Tarassovi	Perepelitsin
L. interrogans	Australis	Australis	Ballico
	Autumnalis	Autumnalis	Akiyami A
	Bataviae	Paidjan	Paidjan
	Bataviae	Bataviae	Van Tienen
	Australis	Bratislava	Jez Bratislava
	Canicola	Canicola	Hond Utrech IV
	Djasiman	Djasiman	Djasiman
	Sejroe	Hardjo	Hardjoprajitno
	Hebdomadis	Hebdomadis	Hebdomadis
	Ictero*	Ictero*	RGA
	Ictero*	Lai	Lai
	Pomona	Pomona	Pomona
	Pyrogenes	Pyrogenes	Salinem
	Sejroe	Wolffi	3705
L. kirschneri	Cynopteri	Cynopteri	3522-C
	Grippotyphosa	Grippotyphosa	Moskva V
	Pomona	Mozdok	5621
L. noguchii	Australis	Muenchen	C90
	Panama	Panama	CZ214K
L. santarosai	Shermani	Shermani	1342-K
L. weillii	Celledoni	Celledoni	Celledoni

Ictero*= Icterohaemorrhagiae

To determine whether isolates were pathogenic or nonpathogenic, two PCR assays were done using the GI:GII set of primers for pathogenic species of *Leptospira* (exception made of *L. kirschneri*) and the B64-1:B64-2 set of primers specific for *L. kirschneri* (Gravekamp *et al.,* 1993), and the MILL1801 and MILL1802 set of primers, specifically designed to amplify a 1,289 bp DNA fragment from the saprophyte *L. biflexa* serovar Patoc *rfb* lipopolysaccharide biosynthetic *locus* (Picardeau *et al.,* 2008). The amplified DNA fragments were visualized on 1 % agarose gels stained with ethidium bromide (5 µg/ 100ml). Analysis of the DNA amplicons obtained was done in an image analyzer (Gel Logic 200, Kodak®).

IS*1533*-based PCR assay

This assay was previously reported by Zuerner *et al.* (1995) and was applied for the identification of the isolates with slight modifications. PCR reactions were done in a 50 µl final volume, containing 25 ng DNA, 10 pmol of the forward EPL-2 and the EPR-2 reverse primers, 200 µmol of dNTPs (Roche®), 2 U of Expand Long High Fidelity DNA-polymerase (Roche®) and 3.5 mM $MgCl_2$. Conditions were as follow: one denaturation step at 94 °C / 5 min, followed by 40 cycles of denaturation at 94 °C / 30 sec, alignment at 55 °C / 50 sec and extension at 68 °C / 6 min, increasing 10 seconds each cycle from cycle 15 up to cycle 40 and a final extension step at

68 °C / 7 min. Amplified DNA fragments were analyzed as previously described.

RFLP of a *gspD-gspE* DNA amplicon

Two degenerate primers were designed: the forward MILL2357 and the reverse MILL2358 primers (Table 2), to amplify a 1,650 bp DNA fragment expanding from *gspD* through to *gspE*, (Mena-Bañuelos, 2009 personal com.). Amplicons were obtained from 7 serovars representative of five pathogenic *Leptospira* species. DNA amplification was done in 50 µl reactions, containing 25 ng DNA, 10 pmol of each primer, 200 µmol dNTPs (Invitrogen®), 2 U of Taq DNA Polymerase (Roche®) and 3.5 mM $MgCl_2$. PCR conditions were: an initial denaturation step at 94 °C / 5 min, followed by 40 cycles of denaturation at 94 °C / 50 sec, alignment at 58 °C / 50 sec, and extension at 72 °C / 110 sec and a final extension step at 72 °C / 7 min. PCR products were digested in a final 25 µl reaction volume with each *Hin*dIII, *Eco*RV, *Bgl*II, *Cla*I and *Kpl*I restriction enzymes (Invitrogen®). Digestions included 1 µl BSA buffer (Invitrogen®) and were incubated at 37 °C for 1 h. DNA digests were analyzed as described for PCR products.

***rrs2* sequencing**

The sequencing of the 16S rRNA gene (*rrs2*), of the isolates was performed at the Fio-Cruz Institute molecular biology laboratory, Salvador, Brazil and further editing and sequence alignment was done with the Sequencher® version 4.6 software. Sequences analysis was done using the Basic Local Alignment Search Tool (BLAST) on line (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to search for similar sequences in the databases (Altschul *et al.*, 1990); alleles were grouped based on the alignments obtained with the BioEdit tool (Hall, 1999). The phylogenic tree was constructed using the Neighbor-Joining method with 2,000 replicates and the Kimura 2-parameters model in the MEGA 4.0 program (Felsenstein, 1985; Saitou and Nei, 1987; Tamura *et al.*, 2007). The accession number for the mice isolates *rrs2* sequence is: HM776722.

Multiple Locus Sequencing Typing (MLST)

MLST on *Leptospira* was firstly reported by Ahmed *et al*. (2006). On the other hand, the website for *Leptospira* MLST (http:// leptospira.mlst.net), operates with a different set of genes (Thaipadungpanit *et al*., 2007). Such MLST schemes are based on the comparative analysis of the sequences of amplicons from the *adk, secY, icdA, lipL32, lipL41* and *rrs2* (Ahmed *et al.*, 2006), or the *glmU, pntA, sucA, tpiA, mreA, pfkB* and *fadD* genes (Thaipadungpanit *et al.*, 2007). In order to determine the suitability of such MLST schemes, we compared both to characterize the mice *Leptospira* isolates.

Table 2. Primer sets used in the present study. Genome position was found on **Leptospira interrogans* serovar Lai, +*L. biflexa* serovar Patoc and $^{\pi}$*L. borgpetersenii* serovar Hardjobovis. No †*L. kirschneri* genome is available up to date.

Primer	Sequence 5´-3´	gene	Size in bp	Genome position	Reference
GI	CTGAATCGCTGTATAAAAGT	**secY*	285	772349-772368	Gravekamp *et al.* (1993)
GII	GGAAAACAAATGGTCGGAAG			772084-772103	
B64-1	ACTAACTGAGAAACTTCTAC	†*nadD*	563	---------	
B64-2	TCCTTAAGTCGAACCTATGA			---------	
MILL1801	CATGAACCAAGGCATACC	+Putative *galE-wecE-1*	1,289	2117074-2117091	Picardeau *et al.*, (2008).
MILL1802	AGAAGAAGTTTAACGGGG			2118347-2118363	
MILL2357	ACNGTNAAYGAYCARGARGC	+*gspD-gspE*	1,707	2350290-2350309	Mena-Bañuelos (2009) personal communication
MILL2358	ACCATDATNACRTCNGGRTC			2348583-3348602	
EPL-2	CTCAACTCTCCAGCACGTTT	$^{\pi}$IS1533 and	variable	variable	Zuerner *et al.* (1995)
EPR-2	CTCGCAAACTCTCGTCCATT	IS1533-like sequences			

Primer sets and PCR conditions were as previously reported, amplicons were submitted for sequencing to Macrogen Inc., Seoul, Korea. Sequence analysis and editing were done with the Sequencher® version 4.6 package. The sequence type for the isolates (ST), was obtained by comparison of their amplicons sequences with a database kindly provided by Prof. Niyaz Ahmed, School of Life Sci., Hyderabad Univ. - Hyderabad, India. Similarly, the ST for Thaipadungpanit *et al.* scheme (2007) was obtained by submission of the sequences of the corresponding alleles to the *Leptospira* MLST website curator.

RESULTS

Isolates, PCR and cross-agglutination identification

23 out of 50 mice (46 %) were considered as positive in the MAT at titers ≥ 1:20. Serovars detected were: Ballum 38 % (19), Canicola 10 % (5) and Australis 2 % (1). The highest antibody titers were 1:80 to serovar Ballum. Three isolates (6 %) were obtained out of the 50 mice captured. Mice 13 and 14 showed MAT titers 1:40 against serovar Ballum, in contrast mouse 28 did not react to any serovar in the MAT. Isolates were identified as pathogenic by the amplification of a 285 bp DNA fragment with the specific set of primers GI:GII (Gravekamp *et al.*, 1993); no amplification was obtained with the B64-1:B64-2 set of primers specific for *L. kirschneri* nor the MILL1801:MILL1802 specific set of primers for non pathogenic leptospires, (data not shown). Serological identification of the isolates was performed by cross-agglutination with the rabbit specific antiserum and a panel of 23 reference strains, where the homologous antiserum showed titers as high as 1:25,600 against the *L. borgpetersenii* serovar Ballum Mus 127 reference strain, the same titer as that showed by the homologous mice isolates.

IS*1533*-based PCR assay

We used the primers EPL-2 and EPR-2 described by Zuerner *et al.* (1995), to obtain the electrophoretic pattern of amplicons for different *Leptospira* serovars (Table 1). Not all serovars showed DNA amplicons with such a primer set, but only those from serovars of *L. borgpetersenii:* Ballum Mus, Ballum Castellonis, Tarassovi Tarassovi; from *L. interrogans:* Bataviae Paidjan, Sejroe Sejroe, Sejroe Wolffi; from *L. noguchii*: Australis Muenchen and from *L. weillii:* Celledoni Celledoni. These resulted in a unique electrophoretic pattern characteristic for each serovar. When comparing the three mice isolates: CRAN13, CRAN14 and CRAN28, all showed the same pattern of amplicons that also was identical to that from serovar Ballum strain Mus 127 (Figure 1).

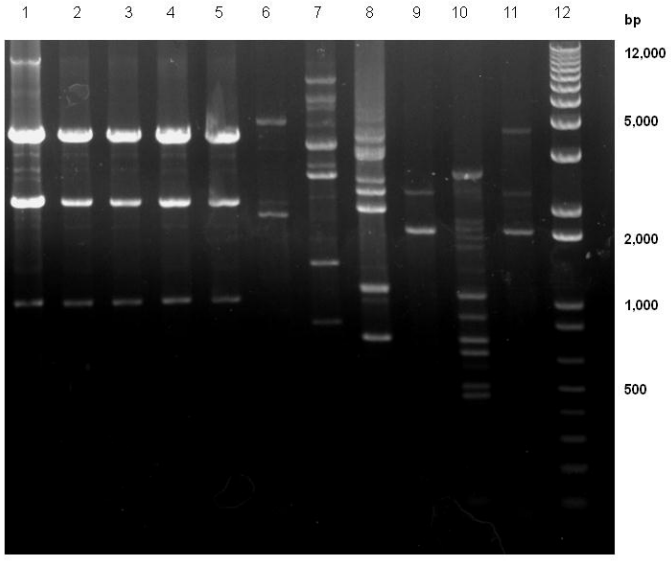

Figure 1. Agarose gel electrophoresis of the IS*1533*-based PCR assay over diverse *Leptospira* serovars. Lane 1: Ballum Castellonis, Lane 2: Ballum Mus 127, Lanes 3 through to 5: Mice isolates CRAN 13, CRAN14, CRAN28, Lane 6: Celledoni Celledoni, Lane 7: Sejroe Sejroe, Lane 8: Tarassovi Tarassovi, Lane 9: Bataviae Paidjan, Lane 10: Muenchen C90, Lane 11: Sejroe Wolffi, Lane 12: Molecular weight marker Invitrogen® 1kb plus. DNA sizes in bp are marked at the right hand of the figure.

RFLP of the *gspD-gspE* DNA amplicon.

A 1,650 bp DNA fragment expanding from *gspD* to *gspE* was amplified with degenerate primers from seven representative strains of five *Leptospira* species and the three mice isolates. *Eco*RV digestion of such an amplicon, showed one restriction site on Grippotyphosa and Panama serovars, and two restriction sites on the five other serovars. *Bgl*II digestion showed one restriction site for Hardjoprajitno, Canicola, Shermani and Ballum serovars and two restriction sites for Grippotyphosa and Panama. On the other hand, *Cla*I did not cut on Hardjoprajitno, Canicola, Grippotyphosa or Panama DNA, but showed one restriction site in serovars Ballum and Castellonis. Serovar Shermani showed a dissimilar pattern of digestion with *Cla*I with at least two restriction sites. Finally, there were no *Hin*dIII nor *Kpl*I restriction sites on any of the amplicons. Analysis of the restriction patterns with the suggested enzymes showed no difference between the serovars Castellonis and Mus of serogroup Ballum and the three mice isolates, CRAN13, CRAN14 and CRAN28 (Figure 2).

***rrs2* sequence analysis**

Analysis of the *rrs2* sequence from the three *Leptospira* isolates confirmed identity with serovars of *L. borgpetersenii*. The Megablast tool was used to search for highly similar sequences, showing 99% similarity with the reported *rrs2* sequence for *L. borgpetersenii* serovar Ballum (GenBank accession no. FJ154591). In addition, the sequences analysis showed the same allelic form among the three isolates; representative sequences from each *Leptospira* species were obtained from GenBank and were aligned with the BioEdit software package. The evolutionary relationship based on the DNA sequencing of the 16S rRNA of the three isolates and the highly similar sequences obtained from Genbank was illustrated by a Neighbor Joining built phylogram, showing distinct clades of the *Leptospira borgpetersenii* serovars (Figure 3).

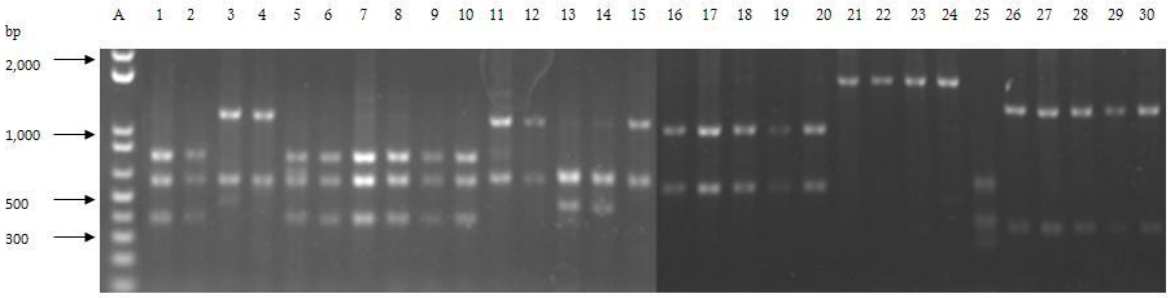

Figure 2. Restriction fragment length polymorphism on the 1,650 bp DNA amplicons expanding *gspD* through to *gspE* of seven different *Leptospira* serovars and the three mice isolates. Lane A: Molecular weight marker Invitrogen® 1kb plus, Lanes 1 through to 10: *Eco*RV digests; Lanes 11 through to 20: *Cla*I digests; Lanes 21 through to 30: *Bgl*II digests. Lanes 1, 11 and 21: Hardjo Hardjoprajitno; Lanes 2, 12 and 22: Canicola Hound Utrecht IV; Lanes 3, 13 and 23: Grippotyphosa Moskva V; Lanes 4, 14 and 24: Panama CZ 214K; Lanes 5, 15 and 25: Shermani 1342 K; Lanes 6, 16 and 26: Castellonis Castellon 3; Lanes 7, 17 and 27: Ballum Mus 127; Lanes 8, 18 and 28: mice isolate CRAN13; Lanes 9, 19 and 29: mice isolate CRAN14; Lanes 10, 20 and 30: mice isolate CRAN28. DNA fragment lengths in bp are shown at the left hand side of the figure.

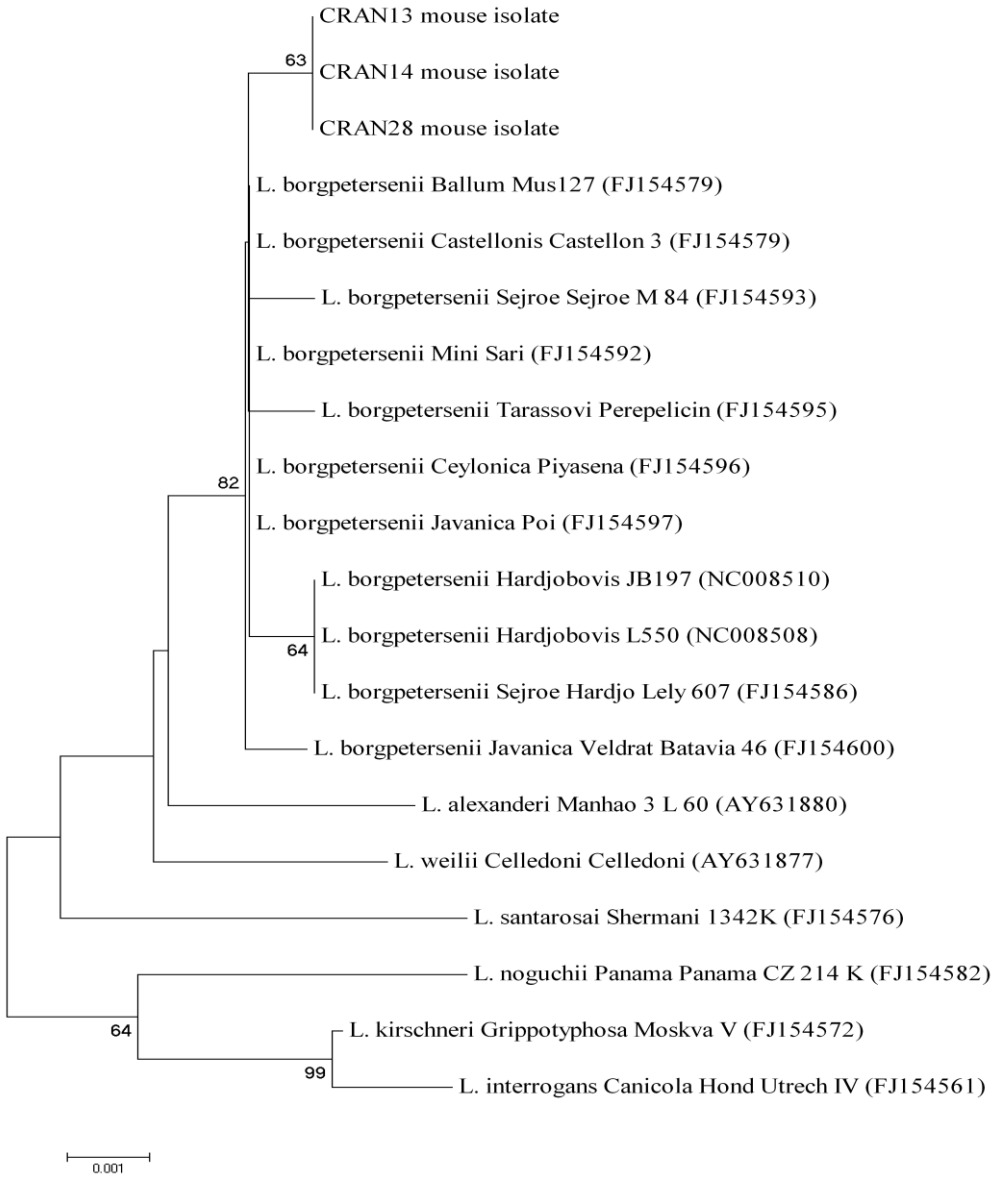

Figure 3. Evolutionary tree derived from the analysis of the 16S rRNA sequences of 17 *Leptospira* strains and the sequences of the three mice isolates obtained in this study. The phylogram was inferred using the Neighbor-Joining method, with 2,000 replicates. There were a total of 1,469 positions in the final dataset.

MLST

Isolates were ultimately characterized as serovar Ballum using the MLST scheme proposed by Ahmed *et al*. (2006), based on the identical sequence type (ST250), showed among the three mice isolates CRAN 13, 14 and 28 and the type strain Ballum Mus 127. In contrast, amplicons were obtained only for the *glmU* and *pntA* in the Thaipadungpanit *et al.* MLST scheme (2007), for this reason, no ST was obtained using such a scheme.

DISCUSSION

Leptospirosis is an emerging disease with a raise in case numbers around the world (Pappas *et al.*, 2008). The disease is considered the most widespread zoonosis worldwide with mortality rates up above 20% in some outbreaks (Bharti *et al.*, 2003; Ko *et al*., 2009). The aim of this study was to evaluate the role of mice as *Leptospira* carriers in a dairy farm in Central Mexico. Previous reports have shown evidence of *Leptospira* infection in domestic animals by isolation of *L. interrogans* serovar Hardjo (Hardjoprajitno) from cattle (Moles *et al.*, 2002), and

more recently the isolation of *L. kirschneri* serovar Grippotyphosa, *L. santarosai* serovars Tarassovi and Mini also from beef cattle (Carmona-Gasca *et al*., 2011). *L.interrogans* serovar Portlandvere has been isolated from a pig kidney (Cisneros *et al.*, 2002); and there are several reports on the presence of *L. interrogans* serovars Canicola (Castillo-Sánchez *et al*., 2007), Portlandvere and Icterohaemorrhagiae (Luna *et al.*, 2008), in dogs. Diagnosis of leptospirosis in both, animals and humans is done traditionally by the MAT. Antibody titers against leptospires such as *L. borgpetersenii* serovars Ballum and Tarassovi, *L.interrogans* serovars Pomona and Bratislava and *L. weilii* serovar Celledoni have also been detected in different domestic animals, nevertheless these serovars have not yet been isolated in Mexico. As far as we know, this is the first report on the isolation of *Leptospira borgpetersenii* serovar Ballum from kidneys of mice (*Mus musculus*) in México.

Rodents mainly rats, have been identified as the most important reservoirs of *Leptospira* (Vijayachari, 2008), and as the potential source of infection for other animal species and the main risk factor for outbreaks in human populations (Gonzalez *et al.*, 2004; Ko *et al.*, 2009). Mice have been previously associated as the source for serovar Ballum infection in some cases of leptospirosis in humans; such as an infection in a large laboratory colony of Swiss albino mice (Stoenner *et al.*, 1958) and an accidental human infection in a laboratory, where mice were the source of infection (Wolff *et al.*, 1949). Important outbreaks caused by serovar Ballum have been identified in Cuba, assuming that rodents were the source of the infection (Gonzalez *et al.*, 2004). In this work, a direct relationship was detected between antibody titers 1:40 in mice 13 and 14 and the isolation of *L.borgpetersenii* serovar Ballum. No titers were found in mouse 28 despite the isolation of the same serovar Ballum from its kidneys.

In Mexico, there are many endemic mice species such as those of the *Baiomys*, *Liomys*, *Peromyscus*, *Osgoodomys*, *Oryzomys*, *Reithrodontomys* genera and introduced mice species such as *Mus musculus*, (Ceballos *et al.*, 2005); hence their presence represents a potential risk factor of leptospirosis to animals and humans. In a study done in the Cozumel Island, Mexico (Sotomayor-Bonilla *et al.*, 2009), a 95.7% of MAT positive reactors (≥1:20), was found in mice of the species *Oryzomys couesi* and *Mus musculus*, a seroprevalence rate more the double when compared with the 46% MAT positive mice we observed in this work.

We confirmed the identity of our isolates as pathogens by the amplification of a 285 bp DNA fragment with the GI:GII specific set of primers for pathogenic *Leptospira* strains (excluding *L. kirschneri*). No amplicon was obtained with the B64-1:B64-2 set of primers specific for *L. kirschneri* strains (Gravekamp *et al*., 1993). In the same manner, the 1,289 bp amplicon specific for non-pathogenic leptospires was not obtained with the MILL1801:MILL1802 set of primers.

The presence of IS*1533* insertion elements have been reported in approximately 40 copies per genome in *L. borgpetersenii* serovar Hardjobovis by Zuerner and Bolin (1988). The IS*1533*-based PCR assay was designed to exploit the presence of different copy numbers of such insertion sequences in the genomes of some *Lep*tospira serovars as a characterization tool (Zuerner *et al.*, 1995). In agreement with that, most of the serovars and strains we used in the present study showed DNA amplicons using the IS*1533*-based PCR assay. In addition, serovars and strains other than those tested by Zuerner *et al.* (1995), such as: Australis Ballico, Autumnalis Akiyami A, Djasiman Djasiman, Grippotyphosa Moskva V, Canicola Hond Utrecht IV, Pomona Pomona, Pyrogenes Salinem, Javanica Veldrat Bataviae 46 and Icterohaemorrhagiae RGA, did not show any evidence of IS*1533* elements using the EPR2 and EPL2 set of primers in the PCR assays. Nevertheless, the assay was discriminative enough to be used for typification of the three mice isolates: CRAN13, CRAN14 and CRAN28; which showed the same IS*1533* pattern as that of the serovar Ballum, Mus 127 reference strain (Figure 1). It is important to state that serovar Ballum was not tested either in the assay originaly reported by Zuerner *et al.* (1995).

The evolutionary relationship of the three mice isolates was determined by the sequence of the 16S rRNA gene and comparison with the GenBank databases. As shown by the phylogram in Figure 3, the highest relationship of the three mice isolates was with *L. borgpetersenii* serogroup Ballum serovars Castellonis Castellon 3 and Ballum Mus 127. Like ribotyping, other studies have been used to identify new *Leptospira* isolates, such as PCR / RFLP strategies applied on different genes such as *flaB* (Kawabata *et al*., 2001), *rrs* and *rrl (*Ralph *et al.*, 1993), and the primer pairs GI:GII or B64-1:B64-2 (Brown and Levett, 1997). In this report, we further achieved to identify the species of three mice isolates using the RFLP of a 1,650 bp *gspE to gspD* DNA amplicon, being this suitable and a straightforward alternative to identify the species of *Leptospira* isolates. Actually, there was no previous information about serovar Ballum restriction sites in this particular *gspD-E* region.

The two most commonly used MLST schemes are those proposed by Ahmed *et al.* (2006) and Thipadungpanit *et al*. (2007). We found that Ahmed *et al*. (2006) MLST scheme was discriminative enough to assign the ST number 250 to the three mice isolates, being those identical to that of serovar Ballum strain Mus 127. In contrast, Thaipadungpanit *et al*. (2007) scheme was not discriminative enough for MLST typification, because five out of seven genes were not amplified. This might be the result of this scheme being intra species directed (*L. interrogans*), in contrast to Ahmed *et al.* (2006) MLST scheme, that is suitable for other *Leptospira* species in addition to *L. interrogans*. In overall, the two more discriminative methods for typification of the mice isolates were the IS*1533*-based PCR assay and MLST that allowed the identification down to the serovar level. Ribotyping on the other hand, showed a 99 % identity between the sequences of the Ballum Mus 127 and Castellonis Castellon 3 reference strains and those of the three mice isolates, resulting in the phylogram shown in Figure 3. However, the sequence of a smaller 462 bp DNA fragment of the same *rrs2* gene selected for the MLST approach, showed a 100 % identity among Ballum Mus 127 and the three mice *Leptospira* isolates.

The isolation of *L. borgpetersenii* serovar Ballum from the kidneys of three mice provides information for future epidemiological studies on the role of mice as reservoirs of pathogenic leptospires and the potential implication on public health and so, the implementation of appropriate control measures. Isolation of *Leptospira* from clinical cases and identification is a task of high importance, this because serodiagnosis of leptospirosis is done based on the serovars present in a specific geographical area (*Ko et al.*, 2009). So that serovar Ballum should be included in the set of serovars used in the MAT for diagnosis in central Mexico.

Acknowledgements

The authors are thankful to Dr. Jorge Ávila García and Mr. Enrique Herrero for the opportunity to perform this study at the “Cantarranas” dairy farm; to Biol. Eliot Zarza for his technical guidance in capturing rodents and to Dr. Rodrigo Medellin for lending us the Sherman-type traps. This project was supported by the PAPIIT IN222806, IN221409 and the MACROPROYECTO No. 7 grants from the Universidad Nacional Autonoma de Mexico; as well by the SEP-CONACyT 83123 grant from the Consejo Nacional de Ciencia y Tecnología (CONACYT). The scholarship No. 165530 granted by CONACYT, México, for the doctorate studies of Carlos Carmona, is highly appreciated.

REFERENCES

Adler, B., de la Peña-Moctezuma, A. 2010. *Leptospira* and Leptospirosis. Veterinary Microbiology. 140:287–296.

Ahmed, N., Devi, S.M., Valverde, M. de L., Vijayachari, P., Machangu, R.S., Ellis, W.A., Hartskeerl, R.A. 2006. Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. Annals of Clinical Microbiology and Antimicrobials. 23:5-28.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. Journal of Molecular Biology. 215:403–410.

Bharti, A.R., Nally, J.E., Ricaldi, J.N., Matthias, M.A., Diaz, M.M., Lovett, M.A., Levett, P.N., Gilman, R.H., Willig, M.R., Gotuzzo, E., Vinetz, J.M. 2003. Leptospirosis: a zoonotic disease of global importance. The Lancet Infectious Diseases. 3:757–771.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim van Dillen, P.M.E., Van der Noordaa, J. 1990. Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology. 28:495-503.

Brown, P.D., Levett, P.N. 1997. Differentiation of *Leptospira* species and serovars by PCR-restriction endonuclease analysis, arbitrarily primed PCR and low-stringency PCR. Journal of Medical Microbiology. 2:173-181.

Carmona-Gasca, C.A., León, L.L., Castillo-Sánchez, L.O., Ramírez-Ortega, J.M., Ko, A., Luna, P.C., de la Peña-Moctezuma, A. 2011. Detection of *Leptospira santarosai* and *L. kirschneri* in cattle: new isolates with potential impact in bovine production and public health. Revista Veterinaria Mexico. 42:277-288.

Castillo-Sánchez, L.O., Roa, R.M.A., De la Peña-Moctezuma, A. 2007. Detection of pathogenic *Leptospira* in asymptomatic dogs. Vth Meeting of the International Leptospirosis Society (ILS). Quito, Ecuador. September 17-20. p61.

Ceballos, G., Arroyo-Cabrales, J., Medellín, R.A., Domínguez-Castellanos, Y. 2005. Lista actualizada de los mamíferos de México.

Revista Mexicana de Mastozoología. 9:21-71.

Cerqueira, G.M., Picardeau, M. 2009. A century of *Leptospira* strain typing. Infection, Genetics and Evolution. 9:760–768.

Cisneros, P.M.A., Ramírez, N.R., Moles, C.L.P., Gavaldón, R.D., Torres, B.J., Rojas S.N. 2002. Aislamiento de *Leptospira* Portland vere a partir de cerdas que abortaron en México. Revista Cubana de Medicina Tropical. 54:52-80.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 39:783-791.

González, M., Batista, N., Machado, M., Savournin, O., Saltarén, A., Sanamé, A., Ochoa, I., Rodríguez, Y., Naranjo, M., Valdés, Y., González, I., Sierra, G. 2004. Caracterización de cepas de *Leptospira* Ballum aisladas de casos clínicos. Inmunidad cruzada en hámsters vacunados con vax-SPIRAL®. Biotecnología Aplicada. 21:77-81.

Gravekamp, C., Van de Kemp, H., Franzen, M., Carrington, D., Schoone, G.J., Van Eys, G.J., Everard, C.O., Hartskeerl, R.A., Terpstra, W.J. 1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. Journal of General Microbiology. 139:1691–1700.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 41:95-98.

Herrmann, J.L., Bellenger, E., Perolat, P., Baranton, G., Saint-Girons, I. 1992. Pulsed field gel electrophoresis of *Not*I digests of leptospiral DNA: a new rapid method of serovar identification. Journal of Clinical Microbiology. 30:1696-1702.

International Committee on Systematics of Prokaryotes. 2008. Subcommittee on the taxonomy of Leptospiraceae. International Journal of Systematic and Evolutionary Microbiology. 58:1049–1050.

Kawabata, H., Dancel, L.A., Villanueva, S.Y., Yanagihara, Y., Koizumi, N., Watanabe, H. 2001. *flaB*-polymerase chain reaction (flaB-PCR) and its restriction fragment length polymorphism (RFLP) analysis are an efficient tool for detection and identification of *Leptospira* spp. Microbiology and Immunology. 6:491-496.

Ko, A.I., Goarant, C., Picardeau, M. 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nature Reviews Microbiology. 7:736–747.

Laras, K., Cao, B.V., Bounlu, K., Nguyen, T.K., Olson, J.G., Thongchanh, S., Tran, N.V., Hoang, K.L., Punjabi, N., Ha, B.K., Ung, S.A., Insisiengmay, S., Watts, D.M., Beecham, H.J., Corwin, A.L. 2002. The importance of leptospirosis in Southeast Asia. American Journal of Tropical Medicine and Hygiene. 67:278–286.

Luna, A.M.A., Moles, C.L.P., Gavaldón, R.D., Nava, V.C., Salazar, G.F. 2008. Leptospirosis canina y su problemática en México. Revista de Salud Animal. 30:1-11.

Majed, Z., Bellenger, E., Postic, D., Pourcel, C., Baranton, G., Picardeau, M. 2005. Identification of variable-number tandem-repeat *loci* in *Leptospira interrogans* sensu stricto. Journal of Clinical Microbiology. 43:539-545.

Moles, C.L.P., Cisneros, P.M.A., Gavaldón, R.D., Rojas, S.N., Torres, B.J.I. 2002. Estudio serológico de leptospirosis bovina en México. Revista Cubana de Medicina Tropical. 1:24-27.

Myers, D.M. 1985. Manual de métodos para el diagnóstico de laboratorio de leptospirosis. Centro Panamericano de Zoonosis. Buenos Aires. OPS, Nota Técnica 30.

Pappas, G., Papadimitriou, P., Siozopoulou, V., Christou, L., Akritidis, N. 2008. The globalization of leptospirosis: worldwide incidence trends. International Journal of Infectious Diseases. 12:351-357.

Perolat, P., Merien, F., Ellis, W.A., Baranton, G. 1994. Characterization of *Leptospira* isolates from serovars hardjo by Ribotyping, arbitrarily primed PCR, and mapped restriction site polymorphisms. Journal of Clinical Microbiology. 32:1949-1957.

Picardeau, M., Bulach, D.M., Bouchier, C., Zuerner, R.L., Zidane, N., Wilson, P.J., Creno, S.,

Kuczek, E.S., Bommezzadri, S., Davis, J.C., McGrath, A., Johnson, M.J., Boursaux-Eude, C., Seemann, T., Rouy, Z., Coppel, R.L., Rood, J.I., Lajus, A., Davies, J.K., Medigue, C., Adler, B. 2008. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. PLoS ONE. 3:e1607.

Ralph, D., McClelland, M., Welsh, J., Baranton, G., Perolat, P. 1993. *Leptospira* species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. Journal of Bacteriology. 4:973-981.

Saitou, N., Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 4:406-425.

Sotomayor-Bonilla, J.J,, Ayanegui-Alcérreca, M.A., Cuarón A.D., Suzán, G., De la Peña-Moctezuma, A. 2009. Seroprevalence of leptospiral serovars among native and exotic rodent species in Cozumel Island, Mexico. VIth Meeting of International Leptospirosis Society (ILS). Cochin, India. September 21-24. pO5.

Stoenner, H.G., Grimes, E.F., Thrailkill, F.B., Davis, E. 1958. Elimination of *Leptospira* Ballum from a colony of Swiss albino mice by use of chlortetracycline hydrochloride. American Journal of Tropical Medicine and Hygiene. 7:4.

Tamura, K., Dudley, J., Nei, M., Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution. 24:1596-1599.

Thaipadungpanit, J., Wuthiekanun, V., Chierakul, W., Smythe, L.D., Petkanchanapong, W., Limpaiboon, R., Apiwatanaporn, A., Slack, A.T., Suputtamongkol Y., White, N.J., Edward, J., Feil, D., Nicholas, P.J., Peacock, S.J. 2007. A dominant clone of *Leptospira interrogans* associated with an outbreak of human leptospirosis in Thailand. PLoS Neglected Tropical Diseases. 1:e56.

Vijayachari, P., Ahmed, N., Sugunan, A.P., Ghousunnisa, S., Rao, K.R., Hasnain, S.E., Sehgal, S.C. 2004. Use of fluorescent amplified fragment length polymorphism for molecular epidemiology of leptospirosis in India. Journal of Clinical Microbiology. 42:3575-3580.

Vijayachari, P., Sugunan, A.P., Shriram, A.N. 2008. Leptospirosis: an emerging global public health problem. Journal of Biosciences. 33:557–569.

Wolff, J.W., Bohlander, H., Ruys, A.C. 1949. Researches on *Leptospira* Ballum: The detection of urinary carriers in laboratory mice. Antonie van Leeuwenhoek. 15:1.

Zuerner, R.L., Alt, D., Bolin, C.A. 1995. IS*1533*-based PCR assay for identification of *Leptospira interrogans* sensu lato serovars. Journal of Clinical Microbiology. 33:3284-3289.

Zuerner, R.L., Bolin, C.A. 1988. Repetitive sequence element cloned from *Leptospira interrogans* serovar hardjo type hardjo-bovis provides a sensitive diagnostic probe for leptospirosis. Journal of Clinical Microbiology. 26:2495-2500.

Submitted August 30, 2010– Accepted February 07, 2012
Revised received June 17, 2012