ARTÍCULO ORIGINAL

ISSN 0325-7541
Revista Argentina de Microbiología (2012) 44: 138-143

In vivo cell aggregations of a recent swine biofilm-forming isolate of *Leptospira interrogans* strain from Argentina

BIBIANA BRIHUEGA[1], LUIS SAMARTINO[1], CARMELO AUTERI [1], AGUSTÍN VENZANO[1], KARINA CAIMI[2*]

[1]Instituto de Patología and [2]Instituto de Biotecnología, CICVyA, INTA Castelar (1712) Hurlingham, Buenos Aires Province, Argentina.
[*]Correspondence. E-mail: kcaimi@cnia.inta.gov.ar

ABSTRACT

Leptospirosis is a zoonosis of ubiquitous distribution caused by spirochetes. Leptospires exist either as saprophytic water-associated organisms or as animal pathogens that can survive in water. Previous works have demonstrated that both saprophytic and pathogenic leptospires are able to produce functional biofilms, which consist of a community of bacteria embedded in an extracellular matrix attached to a surface. This structure is believed to provide protection from environmental aggressiveness. In the present study, we analyzed the capacity of biofilm formation both of a a recent field isolate of *Leptospira interrogans* serovar Pomona obtained from an aborted swine fetus and of the saprophytic *Leptospira biflexa* serovar Patoc. We used light microscopy, immunofluorescence, and scanning electron microscopic examinations on glass and polystyrene plate models to evaluate the process *in vitro*. The ability to form bacterial aggregations *in vivo* was tested using pregnant guinea pigs infected with both strains. We obtained biofilms both on glass and plastic surfaces. Scanning electron microscopic analysis showed differences in the biofilm structure formed by both strains. *L. interrogans* serovar Pomona cell aggregations were observed in placental tissues by light microscopy. Biofilms and cell aggregations are consistent with the life of saprophytic strains in water and could help pathogenic strains to colonize the host and lead to abortion in pregnant animals.

Key words: *Leptospira interrogans*, biofilms, cell aggregations, guinea pigs

RESUMEN

Agregaciones celulares *in vivo* de *Leptospira interrogans* producidas por un aislamiento porcino capaz de formar *biofilm*. La leptospirosis es una zoonosis de amplia distribución causada por el género *Leptospira*. Las leptospiras existen de manera saprófita asociadas a ambientes acuáticos o como patógenos animales que también pueden sobrevivir en el agua. Trabajos previos demostraron que tanto las leptospiras saprófitas como las patógenas tienen la capacidad de formar *biofilms*, que consisten en una comunidad de bacterias embebidas en una matriz extracelular adherida a una superficie. Esta estructura tendría la función de proveer protección contra el medioambiente. En este estudio, analizamos la capacidad de formar *biofilm* en un aislamiento obtenido recientemente de un feto porcino abortado, caracterizado como *Leptospira interrogans* serovar Pomona, y en la bacteria saprófita *Leptospira biflexa* serovar Patoc. Se estudió la formación de *biofilm* en distintas superficies (vidrio y poliestireno), las que se evaluaron por microscopía óptica, inmunofluorescencia y microscopía electrónica de barrido. La capacidad de formar agregaciones bacterianas *in vivo* se evaluó utilizando un modelo de cobayas preñadas infectadas con ambas cepas. Se obtuvieron *biofilms* tanto en las superficies plásticas como de vidrio. La microscopía de barrido mostró diferencias en la estructura del *biofilm* formado entre ambas cepas. Se observaron agregaciones celulares en vasos placentarios de los animales infectados con *L. interrogans* serovar Pomona. Los *biofilms* y las agregaciones celulares son compatibles con la vida saprofítica en el agua y podrían favorecer a los microorganismos patógenos en la colonización del hospedador, lo que podría llevar al aborto en los animales preñados.

Palabras clave: *Leptospira interrogans*, *biofilms*, agregaciones celulares, cobayos

INTRODUCTION

Among the advances in microbiology that have taken place over the past 50 years, one of the most subtle has been the realization of the extent to which microbial growth and development occurs on surfaces in complex communities. Claude Zobell and colleagues noted that aquatic bacteria are more numerous on the solid surfaces of sample containers than as single suspended cells (19). Biofilm formation is an ancient and integral component of the prokaryotic life cycle, and is a key factor for survival in diverse environments. Recent advances have shown that biofilms are

structurally complex, dynamic systems with attributes of both primordial multicellular organisms and multifaceted ecosystems (5, 8). Biofilms are complex structures composed of millions of bacteria that work in a coordinated way either to avoid the phagocytic cells or resist the antibiotic action within the host (7).

Leptospirosis is a zoonosis of ubiquitous distribution caused by the spirochete of the genus *Leptospira*. Nowadays, leptospirosis is identified as a reemerging infectious disease that can be contracted either through direct contact with infected animals or from a contaminated environment due to the capacity of leptospires to survive in soil and water for long periods (16).

Singh *et al.* described a method based on a culture-independent direct amplification and sequencing of 165 subclones from community biofilm 16S rDNA that revealed that 20 % of the identified bacteria corresponded to *Leptospira* spp. (16). Recently, Ristow *et al.* (15) described the formation of biofilms in saprophytic and pathogenic leptospires *in vitro* for the first time. The long-term colonization of the proximal renal tubules of rats by pathogenic leptospires is now believed to proceed via the formation of cell aggregates (2). Thus, biofilm formation may also play an important role in maintaining a chronic carriage of the pathogen *Leptospira interrogans* in animal reservoirs. For these reasons, we tested the ability of a recent local isolate of *L. interrogans* serovar Pomona obtained from an aborted pig to form biofilms *in vitro* and, as a preliminary study, its capacity to form bacterial aggregations in placental vessels of pregnant guinea-pigs.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *L. interrogans* field strain was isolated in our laboratory from a urine sample of an aborted pig belonging to an intensive production herd from Buenos Aires province (Argentina). The isolate was first serotypified as *L. interrogans* serovar Pomona by the microagglutination test (MAT) (4). Genotyping using Variable Number Tandem Repeat (VNTR) and Multilocus Sequence Typing (MLST) rendered that the isolate belong to the most common genotypes found in serovar Pomona, VNTR profile: 4, 1, 6, 10, 8, 2, 3, and sequence type (ST) 37 (9, 12, 17). The saprophytic strain used was *Leptospira biflexa* serovar Patoc. The strains were cultured at 30 °C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco, USA) supplemented with 10 % (vol/vol) rabbit serum, 0.015 % (wt/vol) L-asparagine, 0.001 % (wt/vol) sodium pyruvate, 0.001 % (wt/vol) calcium chloride, 0.001 % (wt/vol) magnesium chloride, 0.03% (wt/vol) peptone, and 0.02 % meat extract (wt/vol) for 5 days until a cellular concentration of 1 x 10^8 bacteria/ml was reached.

Biofilm formation assays

Adherence to glass surfaces: Sterile microscope glass slides (25.4mm x 76.2mm x 1.2mm) and glass coverslips (18 x 18mm) were placed into sterile microscopy glass jars containing EMJH medium inoculated with *L. interrogans* serovar Pomona and *L. biflexa* serovar Patoc separately. The jar caps were sealed to avoid desiccation and cultures were grown at 30 °C. The experiment was performed in triplicate. The slides and the coverslips were removed from the jars at different time intervals (5, 15, 30 and 40 days), rinsed three times with phosphate buffered saline (PBS) 1X in order to eliminate the planktonic non-adherent bacteria and dried out. The slides were observed by light microscopy using the Whartin-Starry (W-S) staining method and Immunofluorescence (IF) using a polyvalent conjugate (USDA, USA) (1, 4). The slides were observed at a magnification of 1000X. The coverslips were used for Scanning Electron Microscopy (SEM) and fixed in 2.5 % glutaraldehyde at room temperature for 1 h. The fixed samples were gradually dehydrated in (V/V) 10 %, 30 %, 50 %, 70 %, 90 % and 100 % ethanol baths, desiccated and carbon evaporated. The samples were then observed under a JEOL JSM 6360LV field emission scanning electron microscope (JEOL Ltd, Japan).

Adherence to polystyrene surfaces: Sterile microscope coverslips (Thermanox, Nunc) were aseptically placed into 24-well polystyrene plates along with 200 µl of the EMJH media inoculated with the corresponding strain and incubated at different time points (5, 15, 30 and 40 days). Eighteen wells were used for the strains and the remaining six were used as control medium without inoculation. The cultures were stopped at the fixed times. The medium was removed and the coverslips were rinsed thoroughly as previously described with PBS 1X, W-S stained and observed by light microscopy and IF as previously described.

Animal experiments

In a previous work, the capacity of the swine isolate to induce abortion was studied in guinea pigs at different stages of pregnancy. Hartley pregnant guinea pigs that weighed 200 g were injected intraperitoneally with 1.5 x 10^7 of the swine isolate or the saprophytic strain in a final volume of 1 ml of EMJH. Animals were monitored daily for signs of illness. All the animals infected with the pathogenic strains aborted. Abortion was observed at the different stages of pregnancy studied (3). The presence of bacterial aggregations in placental vessels was tested only in 40-day pregnant guinea pigs. This stage of pregnancy was used according to the size of placental vessels. Three animals were inoculated intraperitoneally using 1.5 x 10^7 bacteria/ml of the swine isolate typified as *L. interrogans* serovar Pomona and other three were inoculated with *L. biflexa* serovar Patoc. The animals inoculated with the swine isolate were euthanized when the abortion occurred. Placenta tissue was extracted and analized for the presence of leptospiras by light microscopy. The placenta samples were fixed on 10 % formol and paraffinic tissue slides were prepared for the staining. The W-S staining technique was used and the samples were observed under a Leitz-Dialux microscope at a magnification of 1000X. All animal studies were approved by the Animal Research Care Committee of the National Institute of Agropecuarian Technology (INTA).

RESULTS

Biofilm formation on different abiotic surfaces

We compared the ability of a recent swine isolate of *L. interrogans* serovar Pomona and the saprophytic *L. biflexa* serovar Patoc to form biofilms on abiotic surfaces. We used light microscopy (Figure 1) and immunofluorescence (Figure 2) to observe the biofilm formation on glass surfaces and polystyrene coverslips at short (5 and 15 days) and long (30 and 40 days) incubation times. The swine isolate was able to aggregate and colonize most of the surface as compared with the saprophytic strain, in which case some remaining free bacteria could be observed at short incubation times (5 and 15 days) (Figures 1 and 2: A, B), whereas at long incubation times (30 and 40 days) both strains reached the same area of colonization (Figures 1 and 2: C, D). Both strains resisted the washes performed at the different incubation times. The results were the same when the strains were grown in polystyrene coverlips (data not shown).

Scanning electron microscopy was used to analyze the differences in the biofilm structures observed between both strains (Figure 3). The biofilm formed by *L. interrogans* swine isolate was able to cover most of the glass coverslip surface (Figure 3, 1A) as compared with that formed by *L. biflexa* that was set up dispersed on the glass surface (Figure 3, 2A and 2B). *L. interrogans* formed a dense structure where an intricate network of bacteria was observed (Figure 3, 1B and 1C). The structure formed by *L. biflexa* was less compact than that formed by *L. interrogans* and individual leptospires were observed on the surface (Figure 3, 2A).

Cell aggregations in placental tissues

The presence of cells aggregations was tested in 40-day pregnant guinea pigs. One of the three animals inoculated with the *L. interrogans* serovar Pomona aborted at 7 days pi. The necropsy was performed and placental samples were taken for W-S tissue staining. The other two animals aborted at 10 days pi and were euthanized following the same tissue treatment. None of the animals inoculated with *L. biflexa* serovar Patoc aborted, therefore, the animals were euthanized and placenta samples were taken. The W-S tissue staining revealed bacterial aggregations on the vascular endothelium of placental vessels only in the animals inoculated with the pathogenic isolate (Figure 4A). The animals inoculated with the saprophytic strain showed no bacterial aggregations but simple unique bacteria on the vascular endothelium (Figure 4B).

DISCUSSION

Leptospires can live in many different environmental conditions, such as water and soil, and the pathogenic species can also live in the microenvironment within the host. One of the mechanisms that could facilitate these adaptations is their capacity to form and maintain biofilms. Within a biofilm, bacteria become attached to a surface and form complex communities, which are able to interact with each other through intercellular communication and thus allow rapid adaptations to changing environments. The microorganisms within biofilms are notorious for their resistance towards the host immune response and antibacterial agents, as compared to their free-living planktonic counterparts

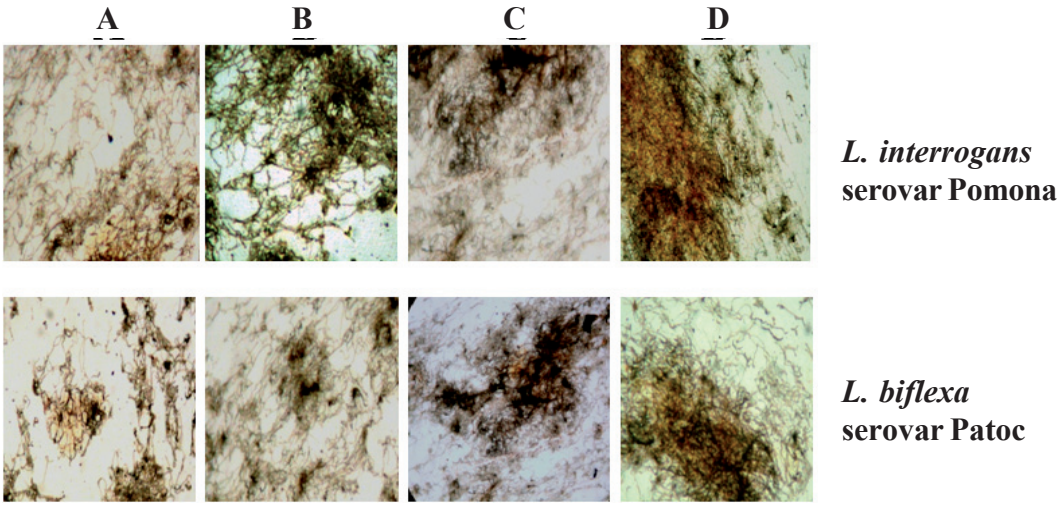

Figure 1. Light microscopy of biofilm formation using the Whartin-Starry staining method. Glass slides were evaluated at different times. A: 5 days; B: 15 days; C: 30 days, and D: 40 days of biofilm formation. Magnification: 1000X.

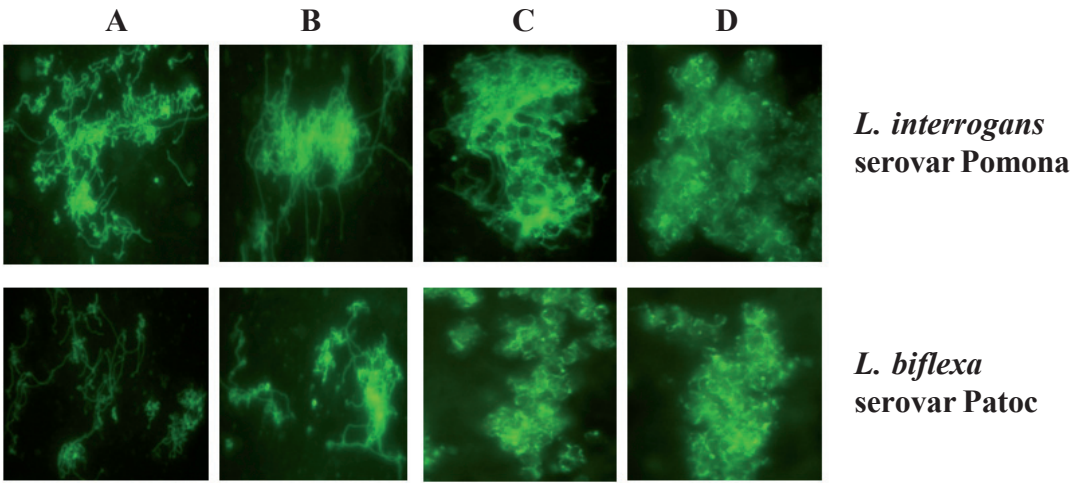

Figure 2. Immunofluorescence assays for assessment of biofilm formation on glass slides. A: 5 days; B: 15 days; C: 30 days, and D: 40 days. Magnification: 1000X.

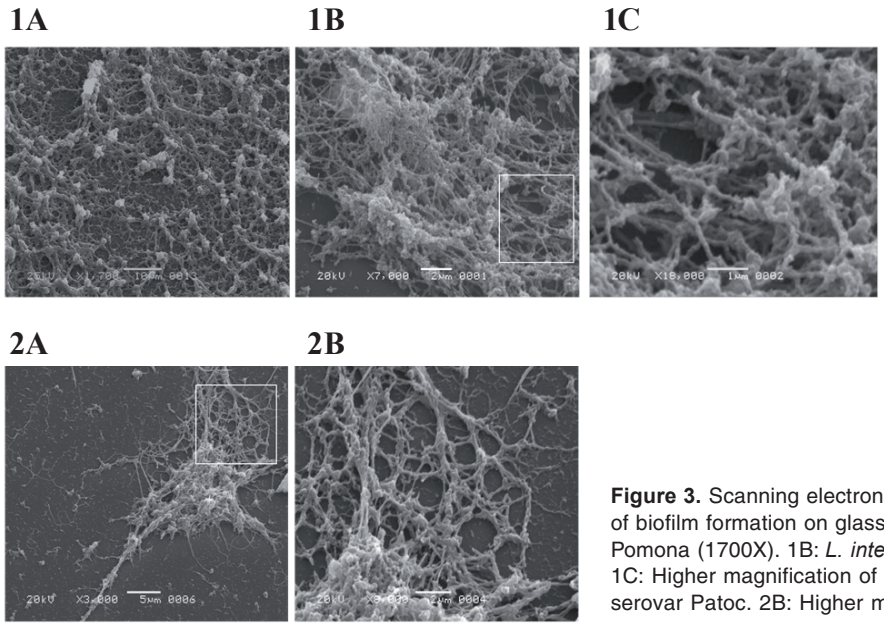

Figure 3. Scanning electron microscopy showing the structure of biofilm formation on glass slides. 1A: *L. interrogans* serovar Pomona (1700X). 1B: *L. interrogans* serovar Pomona (7000X). 1C: Higher magnification of the inset (10 000X). 2A: *L. biflexa* serovar Patoc. 2B: Higher magnification of the inset (8000X).

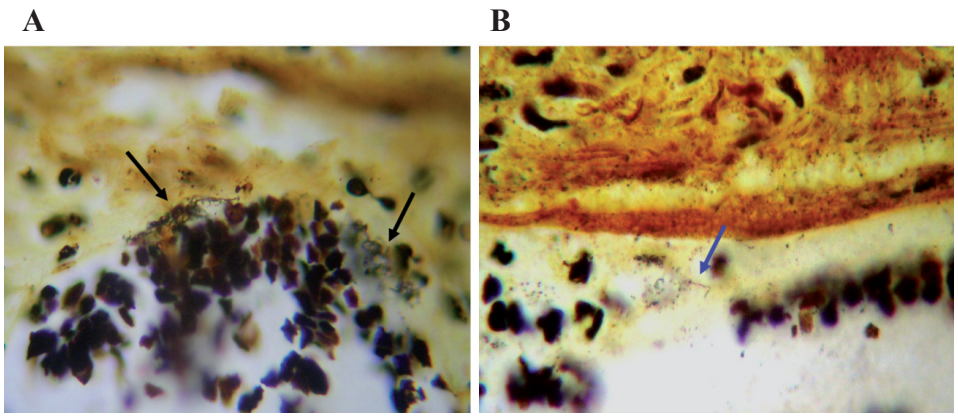

Figure 4. Warthin-Starry staining of placental vessels from infected pregnant guinea pigs. Panel A: *L. interrogans* serovar Pomona. Black arrows show leptospire aggregations. Panel B: *L. biflexa* serovar Patoc. Blue arrow shows no bacterial aggregations (1000X).

(8). Twenty percent of bacterial biofilms are due to *Leptospira* spp., especially in water systems (16). Previous works have reported that *Leptospira* spp. can establish all the stages of a biofilm formation described by Hall-Stoodley *et al.* (8, 15).

According to these previous findings and because of the capacity of pathogenic leptospires to invade different mammalian tissues and to remain as a reservoir for further transmission, we decided to test the ability of a recent swine isolate to form biofilms *in vitro* and cell aggregations *in vivo.* The strain was isolated from a urine sample of an aborted pig and characterized serologically as *L. interrogans* serovar Pomona. In a previous work the strain pathogenicity was tested at different stages of pregnancy in a guinea-pig model and found that all the animals aborted, indicating that this strain was pathogenic and responsible for the pig abortion (3). In this work, the pathogenic isolate was compared with the saprophytic *L. biflexa* serovar Patoc. Light microscopy and immunoflurorescence showed that both strains formed layers of similar density in glass slides at long incubation times (40 days). At short incubation times (5 days), the pathogenic strain developed a thicker layer than that of the saprophytic strain (Figures 1 and 2, A and B). The plastic surfaces analyzed (polysterene slides and coverslips) rendered the same results (data not shown). The biofilms formed by both strains were resistant to the washes performed in both kinds of surfaces at both short and long incubation times. These results differ from those obtained by Ristow *et al.*, who observed that *L. interrogans* was not resistant to the washes performed on the biofilm growing in a plastic surface at short incubation times. The authors suggested that this may be due to the fastidious growth rate of a recent pathogenic isolate growing in a static condition (15). Although a continuous-culture biofilm system was suggested to improve the bacterial attachment to the surface, the results shown in our study suggest that even when a static growth condition is used, a recent pathogenic low passage isolate may establish a complete biofilm structure if long incubation times are allowed and that this structure is resistant to washes. This is consistent with the SEM results obtained in our study where the biofilm structure observed in *L. interrogans* serovar Pomona covered almost all the surface and was thicker than the one formed by *L. biflexa,* where single leptospires were observed when long incubation time was tested.

Previous works have demonstrated that leptospires show evidence of social behavior, which may play a major role in leptospiral survival and transmission. Once the spirochetes reach natural collections of fresh water, they may detect the viscous milieu formed by cell capsules or biofilms from other organisms, upon which *Leptospira* cells aggregate (18). The animal experiments presented here allowed us to demonstrate that the recent pathogenic isolate *L. interrogans* serovar Pomona is able to aggregate in the placental vessel walls of pregnant guinea pigs, in contrast with the results obtained with the saprophyte strain. The presence of aggregates of pathogenic leptospires in placental vessels could be associated with the impairment of the mother-fetus interchange, which could be one of the reasons leading to abortion. The result is consistent with the fact that the different colonization factors that have been identified in *L. interrogans* serovar Copenhageni and serovar Lai genomes (11, 14) are absent in the *L. biflexa* genome (13). These factors correspond to different families of afimbrial adhesins that may contribute to the first steps of infection. The first family includes three paralogous genes, *ligA, ligB* and *ligC,* recently identified in *L. interrogans* and in *Leptospira kirshneri*, which codify for proteins with bacterial immunoglobulin-like (Big) repeat domains (10). The second family of leptospiral adhesin candidates consists of three integrin alpha-like proteins (LIC12259, LIC10021, and LIC13101) (11). These integrins are predicted to be integral membrane proteins, which would support their potential role in ligand-binding interactions on the leptospiral surface and may be involved in the establishment of bacterial aggregations observed *in vivo*.

Although more experiments are needed to prove this last hypothesis, in the present work we applied a successful model for the study of biofilm formation *in vitro* and cell aggregations *in vivo*. This model may be helpful in future studies aimed at understanding the mechanisms developed by pathogenic leptospires at the different stages of pregnancy that lead to abortion.

REFERENCES

1. Appassakij H, Silpapojakul K, Wansit R, Woodtayakorn J. Evaluation of the immunofluorescent antibody test for the diagnosis of human leptospirosis. Am J Trop Med Hyg 1995; 4: 340-3.
2. Athanazio D, Silva E, Santos C, Rocha G, Vannier-Santos M, McBride A, Ko A, Reis M. *Rattus norvegicus* as a model for persistent renal colonization by pathogenic *Leptospira interrogans*. Acta Tropica 2008; 2: 176-80.
3. Brihuega B F. 2008. Pathogenesis of abortion by Leptospires.

Phd Thesis. Buenos Aires, Argentina. University of Buenos Aires.
4. Cole J R, Sulzer C R, Pursell A R. Improved microtechnique for the leptospiral microscopic agglutination test. Appl Microbiol 1973, 6: 976-80.
5. Doyle RJ, editor. Microbial growth in biofilms. Part B: Special environments and physicochemical aspects. Methods in Enzymology. Oxford, UK, Elsevier Inc., 2001, p. 3-469.
6. Faine S. Silver staining of spirochaetes in single tissue sections. J Clin Pathol 1965, 3: 381-2.
7. Ghigo JM. Are there biofilm-specific physiological pathways beyond a reasonable doubt? Res Microbiol 2003, 1: 1-8.
8. Hall-Stoodley L, Costerton J W, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2004, 2: 95-108.
9. 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.
10. Matsunaga J, Barocchi M A, Croda J, Young T A, Sanchez Y, Siqueira I, Bolin C A, Reis M G, Riley L W, Haake D A, Ko AI. Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol Microbiol 2003, 4: 929-45.
11. Nascimento AL, Ko A I, Martins E A, Monteiro-Vitorello C B, Ho P L, Haake D A, Verjovski-Almeida S, Hartskeerl R A, Marques M V, Oliveira M C, Menck C F M, Leite L C C, Carrer H, Coutinho L L, Degrave W M, Dellagostin O A, El-Dorry H, Ferro E S, Ferro M I T, Furlan L R, Gamberini M, Giglioti E A, Go´es-Neto A, Goldman G H, Goldman M H S, Harakava R, Jeronimo S M B, Junqueira-de-Azevedo I L M, Kimura E T, Kuramae E E, Lemos E G M, Lemos M V F, Marino C L, Nunes L R, de Oliveira R C, Pereira G G, Reis M S, Schriefer A, Siqueira W J Sommer P, Tsai S M,. Simpson A J G, Ferro J A, Camargo L E A, Kitajima J P, Setubal J C, Van Sluys M A. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J Bacteriol 2004, 186: 2164-72.
12. Pavan ME, Cairó F, Pettinari, MJ, Samartino L, Brihuega B. Genotyping of *Leptospira interro*gans strains from Argentina by multiple-locus variable-number tandem repeat analysis (MLVA). Comp Immunol Microbiol Infect Dis 2011, 34: 135-41.
13. Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, Wilson PJ, Creno S, Kuczek ES, Bommezzadri S, Davis JC, McGrath A, Johnson MJ, Boursaux-Eude C, Seemann T, Rouy Z, Coppel RL, Rood JI, Lajus A, Davies JK, Médigue C, Adler B. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. PLoS One 2008; 3: e1607.
14. Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, Xu, H, Zhang YX, Xiong H, Lu G, Lu LF, Jiang HQ, Jia J, Tu YF, Jiang JX, Gu WY, Zhang YQ, Cai Z, Sheng HH, Yin HF, Zhang Y, Zhu GF, Wan M, Wang HL, Qian Z, Wang SY, Ma W, Yao ZJ, Shen Y, Qiang BQ, Xia QC, Guo XK, Danchin A, Saint Girons I, Somerville RL, Wen YM, Shi MH, Chen Z, Xu JG, Zhao GP. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. Nature 2003; 422: 888-93.
15. Ristow P, Bourhy P, Kerneis S, Schmitt C, Prevost MC, Lilenbaum W, Picardeau M. Biofilm formation by saprophytic and pathogenic leptospires. Microbiology 2008; 154: 1309-17.
16. Singh R, Stine O C, Smith D L, Spitznagel J K Jr, Labib M E, Williams H N. Microbial diversity of biofilms in dental unit water systems. Appl Environ Microbiol 2003, 69: 3412-20.
17. Thaipadungpanit J, Wuthiekanun V, Chierakul W, Smythe LD, Petkanchanapong W, Limpaiboon R, Apiwatanaporn A, Slack AT, Suputtamongkol Y, White NJ, Feil EJ, Day NPJ, Peacock SJ. A dominant clone of *Leptospira interrogans* associated with an outbreak of human leptospirosis in Thailand. PLoS Negl Trop Dis 2007; 1: 1-6.
18. Trueba G, Zapata S, Madrid K, Cullen P, Haake D. Cell aggregation: a mechanism of pathogenic *Leptospira* to survive in fresh water. Int Microbiol 2004, 7: 35-40.
19. Zobell C E. The effect of solid surfaces upon bacterial activity. J Bacteriol 1943, 46: 39-56.

Recibido: 30/8/2011 – Aceptado: 23/5/2012