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BIOFILM FORMATION BY *Aquaspirillum* spp. AND SAPROPHYTIC *Leptospira* spp. ISOLATED FROM ENVIRONMENTAL SOURCE OF ARGENTINE

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ABSTRACT

Leptospirosis is a zoonotic disease of global distribution, caused by bacteria of the genus *Leptospira*. These spirochetes are living organisms free of mud and water; pathogenic leptospires can survive several days in fresh water when pH and temperature are adequate. During 2016, water samples were collected from Callvú Leovú stream (Azul, Buenos Aires); samples were inoculated in liquid EMJH medium and incubated at 28° C for 90 days. Six isolates of saprophytic leptospires and six of spirils (*Aquaspirillum* spp.) were obtained. The isolates were inoculated in EMJH (liquid and semi-solid) medium and sterile stream water at 4-10° C and 28-30° C; development was observed periodically using dark field microscopy. Both bacteria (alone or together) grew exponentially in first three weeks in all media incubated at 28-30° C; the semi-solid medium was the most efficient at 28-30° C of incubation, and the bacteria remained viable after 16 weeks. At 4-8° C both bacteria remained undetectable but viable in media incubated at 4-8° C for three weeks until the temperature was optimal (thermal stimulation). Leptospires developed in all media used and remained viable for 112 to 168 days (at 4-8° C incubation) in liquid media. The formation of cellular aggregate between *Leptospira* spp. and *Aquaspirillum* spp. was independent at the incubation temperature. These results suggest that *Aquaspirillum* spp. coexists with the genus *Leptospira* in surface waters, and their presence may indicate possible circulation of leptospires.

Keywords: *Leptospira* spp., *Aquaspirillum* spp., biofilm, cell aggregation

INTRODUCTION

Depending on the shape of bacteria, they are classified as cocci (spherical or oval cells), bacilli (rod shaped cells), vibrios (comma shaped curved rods), spirilla (rigid spiral forms), spirochetes (flexible spiral forms), actinomycetes (branching filamentous forms) and mycoplasmas (cell wall deficient forms). Helical bacteria have been found in nature under very diverse circumstances, and spirochetes (*Treponema* spp., *Borrelia* spp. and *Leptospira* spp.) are free-living inhabitants of mud and water.

All *Aquaspirillum* species described in Bergey's Manual of Systematic Bacteriology share the following characteristics: they are all rigid helical cells, except for *Aquaspirillum delicatum* which is vibrio and *Aquaspirillum fasciculus* which is a straight rod. The genus *Aquaspirillum*, with 13 species, was created for all aerobic freshwater spirilla having a low salt tolerance **(Pot, 2006)**. Leptospires are aerobic spirochetes whose cells are flexuous, motile, tightly coiled and have axial flagella; they are gram negative and there is no visual difference between serogroups. Some are pathogenic, though others are harmless freshwater saprophytes; all requiring oxygen (dissolved in water) to survive. The genus Leptospira sp. includes at least 22 species arranged into three large subgroups based on 16S rRNA phylogeny, ten (10) pathogenic species, seven (7) saprophytic species and five (5) intermediate species **(Bourhy *et al.*, 2014; Picardeau, 2017)**. Saprophytic *Leptospira* spp. is free-living environmental microorganisms; however, pathogens leptospires can survive several days in fresh water when pH and temperature are adequate **(Faine *et al.*, 1999; Trueba *et al.*, 2002)**. Each bacterium grows and divides independently of any other bacteria, although aggregates of bacteria (biofilms) have been frequently observed, even with members of different species **(Ristow *et al.*, 2008)**. Biofilm formation has been observed between saprophytic and pathogenic leptospires, *Azospirillum brasiliensis* and pathogenic leptospires, and between *Sphingomonas sp*. whit *Leptospira* spp. **(Barragan *et al.*, 2011; Kumar *et al.*, 2015; Ristow *et al.*, 2008)**. These cellular aggregates would give them protection from dynamic environments and even survive in poor nutrient conditions. In other studies, the ability to form bacterial aggregations in vivo was observed in pregnant guinea pigs infected with Leptospira Pomona **(Brihuega *et al.*, 2012)**.

The present research shows the behaviour and growth of *Leptospira* spp. and *Aquaspirillum* spp. in aqueous and semi-solid fluid (with and without nutrients) incubated at different temperatures, such as the interaction and biofilm-forming of leptospires with environmental bacteria.

MATERIAL AND METHODS

Study area

The stream Callvú Leovú is born in the vicinity of the town of Chillar, Buenos Aires province; and after traveling about 60 km crosses the city of Azul to end at Canal 11 (city of Las Flores), the mentioned channel was built in order to drain the waters of this channel and of other streams towards the Samborombón Bay.

Sample collection and culture

Water samples were collected from Callvú Leovú stream during 2016 years and transported in sterile 500 millilitres glass bottles (Figure 1). Temperature and pH were monitored in the field. Water samples were filtered through a sterile membrane. In this study, a pre-filter technique was applied using Whatman filter paper before filtration through membrane filter with 0.22 µm pore size. All samples were collected in early morning. A sample of the filtered water (one millilitre) was inoculated into Ellinghausen–McCullough–Johnson–Harris (EMJH medium: Difco Laboratories, Detroit Michigan USA) liquid medium without the addition 5-fluorouracil as selective antimicrobial agent. Cultures were incubated by duplicate at 13º C and 28-30º C during 90 days, and bacterial

(*Aquaspirillum* spp. and *Leptospira* spp.) growth was monitored weekly using dark field microscopy. If leptospires or spirilla were not detectable after 90 days of incubation, the sample was considered to be negative.

Production of pure cultures

To obtain pure cultures of leptospires and *Aquaspirillum* spp., liquid and semisolid media of EMJH and Thyoglicollate (pure and mixed) with and without addition of 5-fluorouracil (300 µm/ml) were used, in all cases the pH was 7.2.

Characterization of *Leptospiral* isolates

Multiple Locus Variable number tandems repeat Analysis (MLVA) genotyping

Faine DNA templates were obtained using Chelex Resin-100 (Bio Rad). MLVA was performed using two sets of oligonucleotides specific for pathogenic leptospires (*L. interrogans*, *L. kirschneri* and *L. borgpetersenii*). Oligonucleotides that hybridized to the flanking regions of the VNTR4, VNTR7, VNTR9, VNTR10, VNTR19, VNTR23 and VNTR31 *loci* were used to discriminate strains of *L. interrogans* and oligonucleotides that hybridized to the flanking regions of the VNTR4, VNTR7, VNTR10, Lb4 and Lb5 *loci* were used for *L. kirschneri, L. borgpetersenii* and *L. interrogans* strains **(Majed et al. 2005; Pavan et al. 2011)**. The final volume (50 µl) of each reaction mixture contained polymerase chain reaction (PCR) buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCL), 200 µM deoxynucleoside triphosphates, 2 µM each of the corresponding forward and reverse primers, 2 mM $MgCl_2$, 1.25 U of Taq DNA polymerase (Invitrogen) and 5 µl of DNA template. PCR amplifications were carried out in a Thermo Scientific PxE 0.2 Thermal Cycler, using the following cycling parameters: 94º C for 5 min, followed by 35 cycles of denaturalization at 94º C for 30 s, annealing at 55º C for 30 s and extension at 72º C for 90 s, with a final cycle at 72º C for 10 min. The amplified samples were examined by electrophoresis in ethidium bromide-containing 2% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 100 V for 50 min. Amplified DNA bands were visualized through ultraviolet light exposure (Uvi Tec transiluminator BTS-20.M, Manufacturer UviTec, St. John's Innovation Centre, Cowley Road, Cambridge, England). Amplicon sizes were estimated using CienMarker (Biodynamics) and the GelAnalyzer 2010a program. To calculate repeat copy numbers, the following formula was used: number of repeats (bp) = [fragment size (bp) - flanking regions (bp)]/ repeat size (bp). Repeat copy numbers were rounded down to the closest whole number. If the copy number was less than one, it was rounded to zero.

Sequencing and phylogenetic analysis of Leptospiral strains

PCR targeting the 16S rRNA gene was carried out for bacterial identification. The following primers were used: 5′-GGCGGCGCGTCTTAAACATG-3′ and 5′-GTCCGCCTACGCACCCTTTACG-3′; these primers have the ability to amplify all pathogenic and non-pathogenic species of *Leptospira* sp. **(Djadid *et al.*, 2009)**. After verification of the amplicon by electrophoresis (in an ethidium bromide-containing 2% agarose gel) and visualization upon UV light exposure, PCR products were purified using a commercial kit (Embiotech). The sample was sequenced at the Institute of Biotechnology, National Institute of Agricultural Technology (Argentina) using a 3130xl Genetic Analyzer (Applied Biosystems). For alignment and construction of the phylogeny, the program MEGA version 6.06 was used **(Tamura *et al.*, 2013)**. The dendogram basing in partial sequences of the 16S rRNA gene was constructed using Neighbour-joining with a bootstrap of 100.

Characterization of *Aquaspirillum* sp. isolates

Real-time PCR amplification of the 16S rRNA gene was performed in a Rotor Gene Q thermocycler (Qiagen, Hilden, Germany) in a final volume of 20 µl using EvaGreen as intercalating fluorescent dye (KAPA FAST, Biosystems, Woburn, USA). Generic primers p201 (5′-GAGGAAGGIGIGGAIGACGT-3′) and p1370 (5′-AGICCCGIGAACGTATTCAC-3′) were used **(Tseng *et al.*, 2003)**. Primers were synthesized at Operon (Huntsville, Alabama, USA). Different PCR amplification conditions were tested, by changing variables such as temperature of annealing, primer concentration, number of cycles, DNA concentration and final reaction volume, in order to produce suitable fluorescence levels for HRM analysis. During the validation process the PCR products were run on agarose gels to check the size of the amplicons. PCR amplifications were carried out in a Rotor Gene Q thermocycler (Qiagen, Hilden, Germany) in a final volume of 20 µl using EvaGreen as intercalating fluorescent dye (KAPA FAST, Biosystems, Woburn, USA). The selected cycling program consisted of an initial denaturation of 2 minutes at 95° C, and 45 cycles of 94° C 10", 60° C 15", and 72° C 15". PCR products were purified and sequenced.

Bacterial strains and growth conditions

The leptospiral and spirilla strains used in this study were isolated from water samples of Callvú Leovú stream during 2016 years. Bacterial cells in logarithmic phase (1-2 x 10^8 cells / ml) were cultured by quadruplicate in EMJH medium (liquid and semi-solid) and sterile stream water; all tubes were incubated at two temperatures ranges (4-10º C and 28-30º C) during 20 weekend and the growth was monitored periodically using dark-field microscopy. To perform this experiment, three groups were formed: "a" (Leptospires alone: *Leptospira* spp. strain lepto106), "b" (Spirilla alone: *Aquaspirillum* spp. strain aquas106), and "c" (*Leptospira* spp. strain lepto106 whit *Aquaspirillum* spp. strain aquas106 at equal concentrations).
The concentration considered optimal was standardized by direct microscopy dark field using Neubauer chamber.

RESULTS AND DISCUSSION

Bacterial strains isolates

During the period April-December 2016 six leptospiral strains and six spirilla strains were obtained. All leptospiral isolates (strain lepto104, strain lepto106, strain lepto109, strain lepto110, strain lepto113 and strain lepto114) were negative by Multiple-Locus Variable-number tandem repeats Analysis (MLVA), however, molecular identification by 16S rRNA gene sequences verified that strain lepto106 (used in this experiment) were identified as non-pathogenic leptospires whit sequence similarities of 99% using Blast (results not shown), and closely related to the species *L. yanagawae* and *L. meyeri*. All spirilla bacteria isolates (strain aquas106, strain aquas108, strain aquas109, strain aquas110, strain aquas113 and strain aquas114) were identified as *Aquaspirillum* spp. by molecular identification by 16S rRNA gene sequences.
In moment of waters samples recollection in Callvú Leovú stream, we register temperature and pH; the results obtained can be seen in Table 1.
Leptospires such as spirilla developed both in tubes incubated at 13° C and at 28-30° C. In a sample of water obtained in month of January only the growth of *Aquaspirillum* spp. was observed in media incubated at 13º C. Generally, in all tubes first the presence of *Aquaspirillum* spp. (cells of 5-10 µm in length and less than 0.22 µm of diameter characterized by the presence of 3-5 turns and a characteristic movement) was observed, and a few days later the leptospires appeared, characterized by their flexuous motility and morphology typical of the genus *Leptospira* spp. (cells of 10–20 µm in length and less than 0.22 µm of) under dark field microscopy (Table 2). All strains isolation was maintained in liquid and semisolid EMJH media.
diameter

Figure 1 Callvú Leovú stream, Azul, Buenos Aires province, Argentina. Area of collection of water samples.

Table 1 Bacterial strains isolate from waters samples in Callvú Leovú stream, Azul, Buenos Aires province, Argentina.

	Stream water		Growth in EMJH medium (Days)			
Months	**Tº**	**pH**	***Leptospira* spp.**	***Aquaspirillum* spp.**	***Leptospira* spp.**	***Aquaspirillum* spp.**
April	15	7.8	7	0	7	0
May	12	7.8	7	0	8	6
June	12	7.4	0	12	0	0
August	14	7.7	10	8	6	4
September	17	7.6	18	5	4	13
November	16	8.6	14	7	7	4
December	23	8.3	4	4	5	0

Legend: Tº- temperature

Obtaining pure cultures of leptospiral and spirilla bacteria

The EMJH media with the addition of 300 µg / ml of 5-fluorouracil were used to obtain the growth of leptospires in absence of spirilla bacteria. To obtain growth of spirilla bacteria in absence of leptospires, Thioglycollate media with addition

of EMJH (10%) and reverse were used. In Thioglycollate media (with 10% EMJH medium) growth of *Aquaspirillum* spp. (3 x 10^7 cells / ml) without the presence of leptospires, was observed on the second day of incubation at 28-30° C; in this media biofilm-forming of *Aquaspirillum* spp. and changes in cell structure (length and number of spires increased) were found. Under these conditions they remained viable for more than 30 weeks. In EMJH medium (with 10% Thioglycollate media) growth of *Aquaspirillum* spp. was observed during the second day of incubation at 28-30° C; biofilm-forming and changes in cell structure were not observed.

Table 2 Differential characteristics of leptospires and spirilla bacteria

Phenotypic characteristic	*Leptospira* spp. strain lepto106	*Aquaspirillum* spp. strain aquas106
Growth at 13°C	X	X
Growth at 28-30°C	X	X
Growth at 4-8°C	X	X
Growth at pH <7.2	-	-
5-fluorouracil resistance	X	-
Growth in EMJH medium	X	X
Growth in Thioglycollate medium	-	X
Helicoidally form	Flexuous helical cells	Rigid helical cells
Cells dimensions	10–20 µm in length and less than 0.22 µm of diameter	10-60 µm in length and less than 0.22 µm of diameter
Motility	By axial filaments	By polar flagellum

Incubation and growth at 28-30º C

a) Leptospires alone (*Leptospira* spp. strain lepto106): In liquid EMJH medium leptospires reached their maximum development (3 x 10^7 cells / ml) in the third week of incubation, and from the fourth to the seventh a plateau phase was observed with approximately 2.5 x 10^6 cells / ml; then declined and viable cells were undetectable after 12 weeks of incubation (Figure 2A). In contrast, in semi-solid EMJH medium the maximum development (2.5 x 10^6 cells / ml) was observed from the second week of incubation, and remained stable for up to 12 weeks (Figure 2B). In watercourse leptospires behaved similarly to those observed in liquid EMJH media (Figure 2C).

b) Spirilla alone (*Aquaspirillum* spp. strain aquas106): In liquid EMJH media spirilla bacteria showed a progressive growth, reaching 3 x 10^7 cells / ml in the second week of incubation. In the first three weeks of incubation cells were observed to be larger with increase in the number of spires. After the third week spirilla bacteria remained viable for up to 12 weeks (Figure 2A). In semi-solid EMJH medium, *Aquaspirillum* spp. increased 3 x 10^7 cells / ml rapidly in the first week of incubation. From the seventh week *Aquaspirillum* spp. enter a plateau phase up to 12 weeks with approximately 2.5 x 10^6 cells / ml (Figure 2B); then in death phase cell mobility was not observed. In this fluid, during the first three weeks of incubation, changes in cellular structure (length and number of spires increased) were observed. In stream water *Aquaspirillum* spp. showed behaviour like that liquid EMJH media (Figure 2C).

c) Co-culture (*Leptospira* spp. strain lepto106 and *Aquaspirillum* spp. strain aquas106): in liquid EMJH medium both bacteria behaved in a similar way, increasing from 2.5 x 10^6 cells / ml to nearly 5 x 10^6 cells / ml during the first three weeks. Approximately 2.5 x 10^6 cells / ml, was constant for several weeks. In these medium small cells aggregates until week 14 were visible (Figure 4A); spirilla bacteria maintained mobility but changes in cellular structure (increase in length and number of turns) were observed. In semi-solid EMJH medium, leptospires increased to approximately 5 x 10^6 cells / ml) in the first week of incubation, however from the third week spirilla bacteria maintained similar cells concentrations (Figure 3B). In this media cellular aggregates on the surface of media were observed until the sixth week (Figure 4). In stream water *Aquaspirillum* spp. strain aquas106 growth more efficiently than EMJH media, increasing to 3 x 10^7 cells / ml in the first week up to week five of incubation. In this fluid leptospires growth similarly to that EMJH media (Figure 3C).

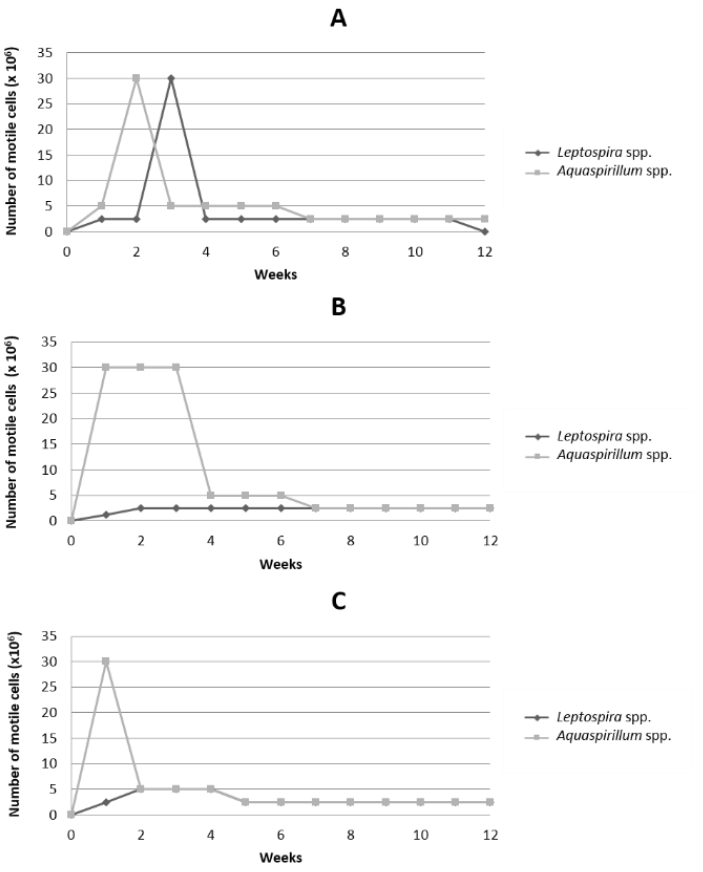

Figure 2 Development of *Leptospira* spp. strain lepto106 and *Aquaspirillum* spp. strain aquas106 alone in liquid EMJH medium (A), semi-solid EMJH medium (B) and stream water (C) incubated at 28-30° C.

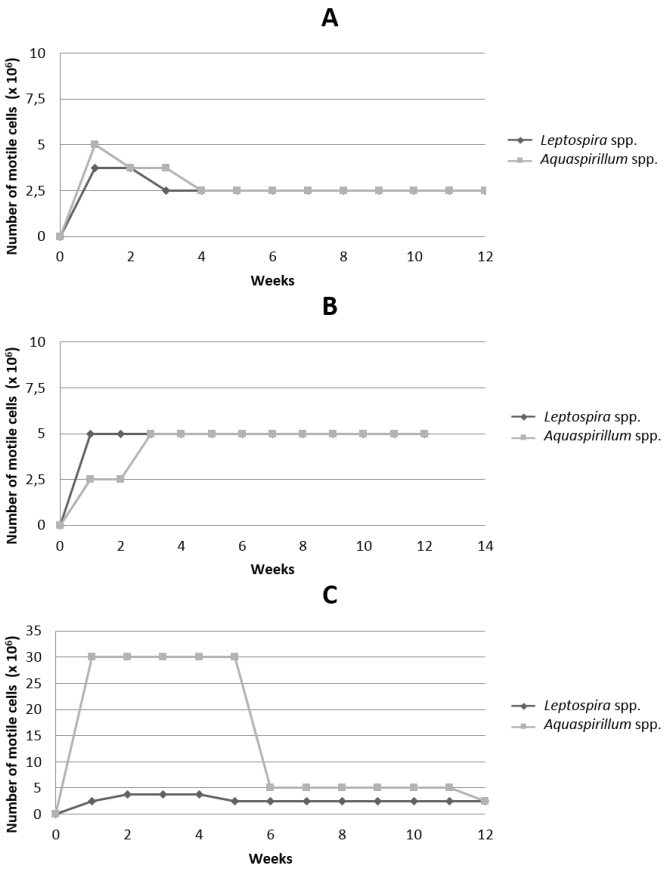

Figure 3 Co-culture of leptospires (*Leptospira* spp. strain lepto106) and spirilla (*Aquaspirillum* spp. strain aquas106) in liquid EMJH medium (A), semi-solid EMJH medium (B) and stream water (C) incubated at 28-30° C.

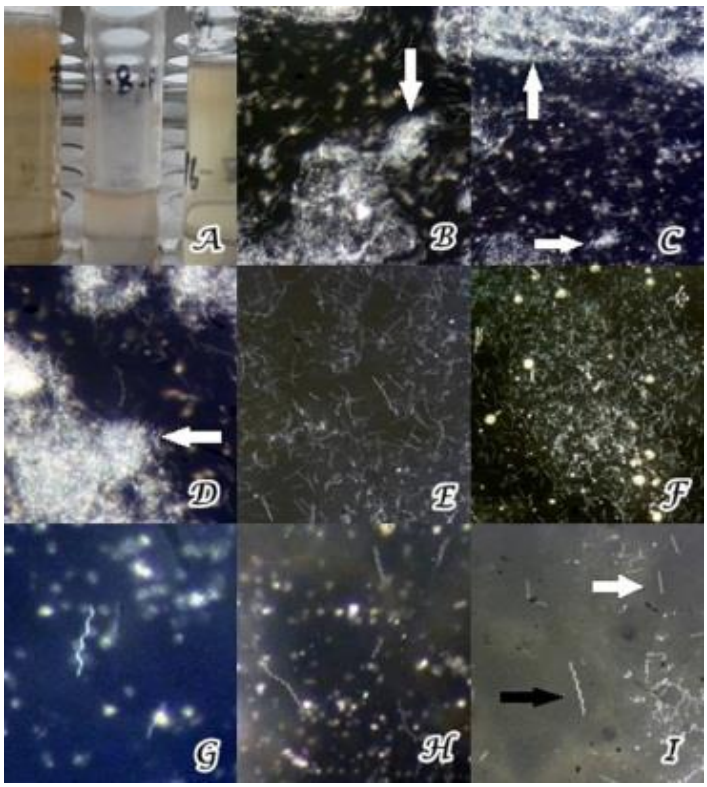

Figure 4 Cell aggregation and formation of biofilm between leptospires and *Aquaspirillum* spp. in semi-solid fluid incubated at 4° C. (A) surface-attached biofilm in air-liquid interface. (B, C and D) aggregates between *Leptospira* spp. strain lepto106 and *Aquaspirillum* spp. strain aquas106 (white arrows) at 40x and 100x magnification. (E and F) dark field microscopy of spirilla bacteria and free leptospires in sectors with no cell aggregates at 100x and 40x magnification, respectively. (G and H) helical shape of *Aquaspirillum* spp. with variable number of spiras at 200x and 100x magnification, respectively. (I) leptospires (white arrows) and *Aquaspirillum* spp. (black arrows) at 100x magnification.

Incubation and growth at 4-8ºC

a) Leptospires alone (*Leptospira* spp. strain lepto106): leptospires developed at similarly in all media used. Although no significant development (2.5 x 10^6 cells / ml) was observed; bacteria were viable and mobility after 12 weeks of incubation (Figure 5A, B and C). In liquid media leptospires increased their size, and even their hooks were more notorious under observation by dark field microscopy.

b) Spirilla alone (*Aquaspirillum* spp. strain aquas106): cells were undetectable in the first three weeks of incubation in all media used, however, when tubes were incubated at 28-30° C for one week, spirilla bacteria increased to approximately 3 x 10^7 cells / ml (Figure 6A and B) and 5 x 10^6 cells / ml (Figure 6C). In semi-solid EMJH media *Aquaspirillum* spp. strain aquas106 showed a more extensive stationary phase than liquid media, in addition cells with atypical mobility and shape were observed. No viable cells were seen after the eighth week in stream water media; however, in viscous media 2.5 x 10^6 cells / ml were observed.

c) Co-culture (*Leptospira* spp. strain lepto106 and *Aquaspirillum* spp. strain aquas106): in liquid media (EMJH and stream water) no development was observed in the first 3 weeks, however, when tubes were incubated at 28-30° C for one week, spirilla (3 x 10^7 cells / ml) and leptospires (2.5 x 10^6 cells / ml) were observed (Figure 7A and C), remaining viable after 10 weeks in liquid EMJH medium. However, in stream water (after incubation at 28-30º C) 2.5 x 10^6 leptospires / ml and 5 x 10^6 spirilla / ml were found, from the fifth week the number of cells / ml was reversed. In semi-solid EMJH media leptospires increased to 3 x 10^7 cells / ml and spirilla to 2.5 x 10^6 cells / ml in the first week of incubation at 4-8° C (Figure 7B), this concentration was constant after 16 weeks of incubation. The formation of cellular aggregate between leptospires and spirilla bacteria was observed from the first week of incubation (Figure 4).

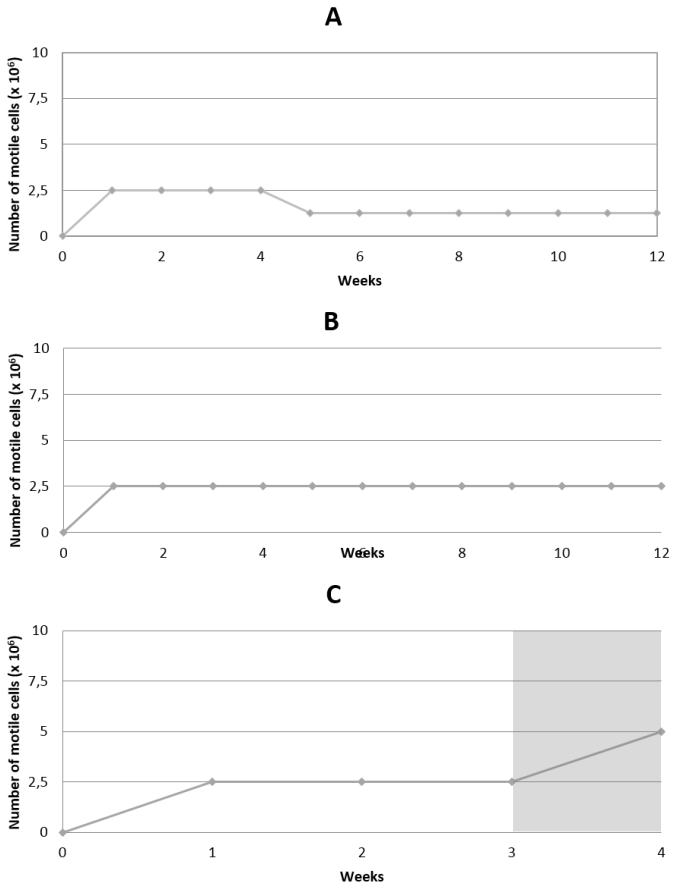

Figure 5 Development of *Leptospira* spp. strain lepto106 in liquid EMJH medium (A), semi-solid EMJH medium (B) and stream water (C) incubated at 4-8° C. In the grey column the period of incubation at 28-30° C is observed.

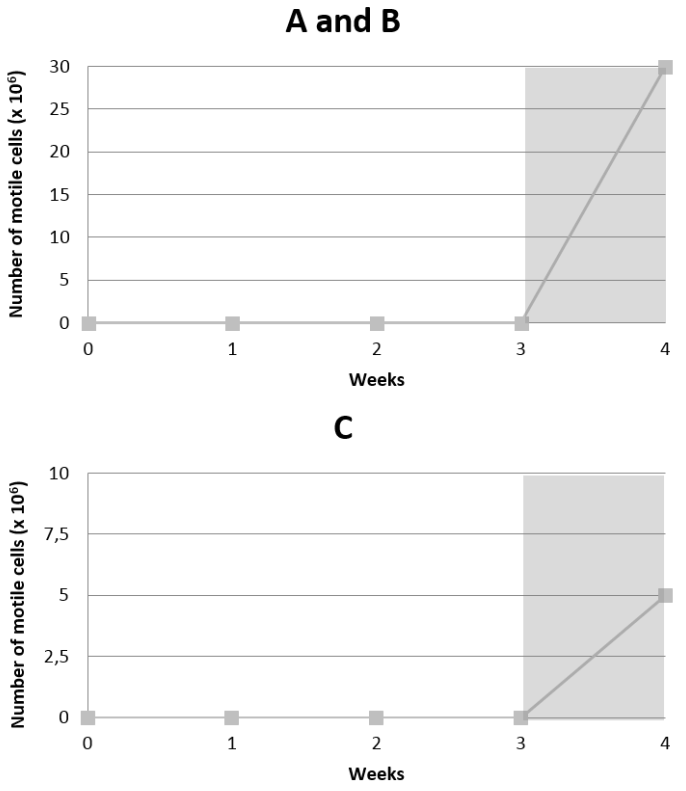

Figure 6 Development of *Aquaspirillum* spp. strain aquas106 in liquid EMJH medium (A), semi-solid EMJH medium (B) and stream water (C) incubated at 4-8° C. In the grey column the period of incubation at 28-30° C is observed.

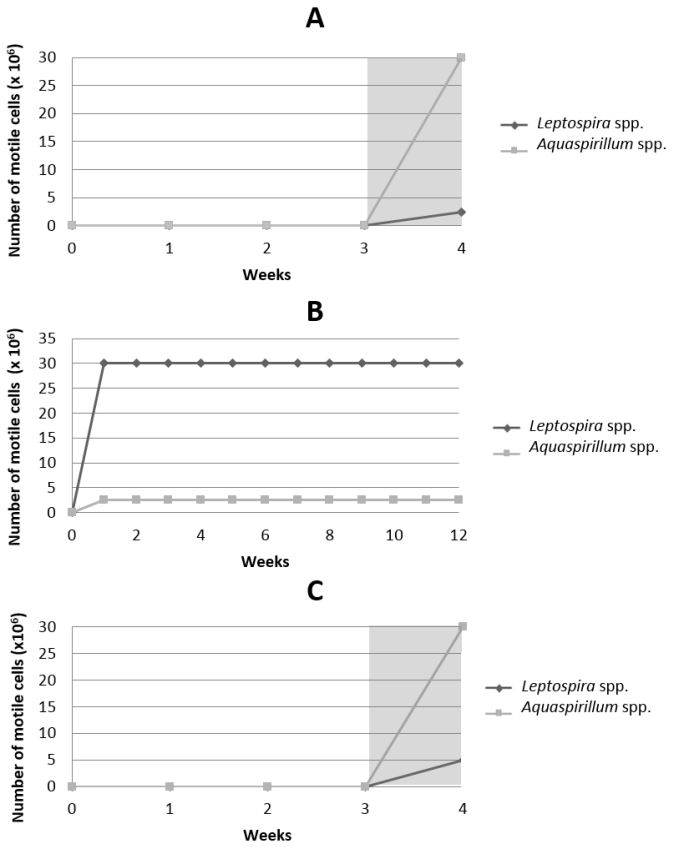

Figure 7 Co-culture of leptospires (*Leptospira* spp. strain lepto106) and spirilla (*Aquaspirillum* spp. strain aquas106) in liquid EMJH medium (A), semi-solid EMJH medium (B) and stream water (C) incubated at 4-8° C. In the grey column the period of incubation at 28-30° C is observed.

In water samples from Callvú Leovú stream, spirilla and saprophytes leptospires were isolated; the growth in liquid EMJH media was on average of 4.6 days at 13° C and 6.9 days at 28-30° C. The pH recorded in water samples was always above 7.2, being optimum for the growth of helical bacteria; in winter period the temperature of stream water was not lower than 11.5° C, and did not inhibit the bacterial development in specific media. Ours results suggest the importance of 5-fluorouracil as specific antimicrobial when leptospires are to be isolated from surface water samples. Spirilla (*Aquaspirillum* spp.) they are rigid helical bacteria not retained by membranes filter whit a pore diameter size of 0.22 µm. These bacteria could be confused with leptospires at low magnification under dark field microscopy. The differential characteristics between *Leptospira* spp. and *Aquaspirillum* spp. are detailed in the table 2. The viability of leptospires and spirilla bacteria in all media used according to incubation temperature is detailed in the table 3.

Table 3 Viability of the bacteria in the media used according to incubation temperature, using dark field microscopy

Media	*Leptospira* spp.		*Aquaspirillum* spp.		*Leptospira* spp. + *Aquaspirillum* spp.	
	4-8 °C	28-30 °C	4-8 °C	28-30 °C	4-8 °C	28-30 °C
Liquid EMJH medium	168	84	77	84	140	98
Semi-solid EMJH medium	168	168	84	98	217	168
Stream water	112	98	56	84	105	98

In specific media for the isolation of leptospires as in stream water, spiral bacteria always reached a higher number of cells in the first week of incubation at 28-30° C compared to leptospires. Spirilla bacteria was undetectable in the first three weeks of incubation at 4-8° C in all media used, however, in a face of thermal stimulus (incubation at 28-30° C for one week) cells increased to approximately 3 x 10^7 cells / ml, and in semi-solid EMJH medium to remain viable after 12 weeks. Spirilla bacteria and leptospires were able to remain viable for three weeks at low temperatures until the environment conditions are optimal.

Leptospires developed in all media used and remained motile for 112 to 168 days (at 4-8° C of incubation) in stream water and liquid EMJH medium respectively; however semi-solid EMJH media was more efficient at 28-30° C. These results are like those observed by **Trueba *et al.*, (2004)** who described the survival of leptospires for 110 days (aqueous media) and 347 days (semi-solid medium) in distilled water **(Trueba *et al.*, 2004).** In liquid EMJH medium viable cells of leptospires were undetectable after 84 days of incubation at 28-30° C, possibly by depleting nutrient medium.

Leptospires and spirilla bacteria (co-culture) growth in liquid EMJH media incubated at 28-30° C, in this medium, *Aquaspirillum* spp. strain aquas106 maintained mobility for several weeks, although changes in cell structure (increase in length and number of turns) were observed. The co-culture in liquid media (EMJH and stream water) incubated at 4° C, *Aquaspirillum* spp. strain aquas106 did not develop during three weeks. In semi-solid EMJH media, *Leptospira* spp. strain lepto106 showed more cell numbers / ml in the first week of incubation respect to *Aquaspirillum* spp. strain aquas106, and this concentration was constant after 16 weeks. In stream water *Aquaspirillum* spp. strain aquas106 showed more efficient growth than EMJH medium.

In semi-solid EMJH medium, cell aggregates between leptospires and spirilla bacteria were observed on the surface, which persisted up to the sixth week. **Ristow *et al.*, (2008)** observed that *L. interrogans* serovar Lai strain Lai 56601 formed a halo attached to the wall of glass tubes at the air–liquid **(Ristow *et al.*, 2008)**.

Cell aggregation and formation of biofilm between *Leptospira* spp. strain lepto106 and *Aquaspirillum* spp. strain aquas106 was observed in semi-solid fluid incubated at 4° C as 28-30° C. In our study, the formation of cellular aggregate between leptospires and spirilla bacteria was independent at incubation temperature. Other studies showed the ability of *L. biflexa* to form biofilm at three different temperatures **(Ristow *et al.*, 2008)**. Viscosity may favour the aggregation of leptospires by providing a matrix that holds the cells together, facilitating motility and therefore chemotaxis **(Trueba *et al.*, 2004)**. Cell aggregation may be a mechanism that facilitates the adaptation of leptospires to different environmental conditions. Two types of biofilm architecture were observed by **Ristow *et al.*, (2008)**, one consisting of large, distinct mound-shaped microcolonies (*L. interrogans*) and the other showing smaller microcolonies with a flatter structure that were linked together by a complex network of *L. biflexa* **(Ristow *et al.*, 2008)**. This mechanism has been observed in saprophytic leptospires as well as in pathogens leptospires **(Barragan *et al.*, 2011; Brihuega *at al.*, 2012; Ristow *et al.*, 2008; Trueba *et al.*, 2004)**.

CONCLUSION

In natural water sources there is a great diversity of environmental bacteria that could interact with the leptospires. This interaction has been with *Sphingomonas consortium* and *Azospirillum brasilense* **(Barragan *et al.*, 2011; Kumar *et al.*, 2015)**. Leptospires and spirilla bacteria share similar habitats in nature, the presence of *Aquaspirillum* spp. in water would help to increase the average life of leptospiras in the environment. All isolates were negative by Multiple-Locus Variable-number tandem repeats Analysis (MLVA), however, molecular identification by 16S rRNA gene sequences verified that all isolates were identified as non-pathogenic leptospires. Further studies will aim to meet serogroups circulating leptospires in surface waters.

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