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 4×10^6 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), actinomyces (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 delicaturn* which is vibrio and *Aquaspirillum fasciculus* which is a straight rod. The genus *Aquaspirillum*, with 13 species, was created for all aerobic freshwater spirailla 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 165 rRNA phylogeny, ten (10) pathogenic species, seven (7) saprophytic species and five (5) intermediate species (Bourry et al., 2014; Picardeau, 2017). Saprophytic *Leptospira* spp. is free-living environmental microorganisms; however, pathogen leptospires can survive several days in fresh water when pH and temperature are adequate (Faune 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 brasilienensis* 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 before filtration. 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 13° C and 28-30° 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 Thyoglicolite (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 tandem repeats Analysis (MLVA) genotyping
A RA E

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. borgpertsonii). 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. borgpertsonii 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 μM each of the corresponding primer, 2 μM MgCl₂, 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: 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°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-20-0, 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'-GGCGCGCCGTTCTAAACATG-3' and 5'-GTCGGCTTACGGACCTTTACG-3'; these primers have the ability to amplify all pathogenic and non-pathogenic species of Leptospira sp. (Djidjé 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 by direct microscopy dark field using Neubauer chamber.

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 10⁸ 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 (4-10°C and 28-30°C) during 20 weekend and the growth was monitored periodically using dark-field microscopy. To perform this experiment, three groups were formed: “a” (Leptospira 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 whist sequence similarities of 99% using Blast (results not shown), and closely related to the species L. yangugwerei 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 13°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.

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.

<table>
<thead>
<tr>
<th>Months</th>
<th>Stream water</th>
<th>Growth in EMJH medium (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>April</td>
<td>15 7.8 7</td>
<td>Leptospira spp. Aquaspirillum spp. 0 7 0</td>
</tr>
<tr>
<td>May</td>
<td>12 7.8 7</td>
<td>Leptospira spp. Aquaspirillum spp. 0 8 6</td>
</tr>
<tr>
<td>June</td>
<td>12 7.4 0</td>
<td>Leptospira spp. 0 12 0</td>
</tr>
<tr>
<td>August</td>
<td>14 7.7 10</td>
<td>Leptospira spp. Aquaspirillum spp. 6 4 13</td>
</tr>
<tr>
<td>September</td>
<td>17 7.6 18</td>
<td>Aquaspirillum spp. 5 4 13</td>
</tr>
<tr>
<td>November</td>
<td>16 8.6 14</td>
<td>Aquaspirillum spp. 7 7 4</td>
</tr>
<tr>
<td>December</td>
<td>23 8.3 4</td>
<td>Aquaspirillum spp. 4 5 0</td>
</tr>
</tbody>
</table>

Legend: T° 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, Thioylcollate media with addition...
of EMJH (10%) and reverse were used. In Thioglycollate media (with 10% EMJH medium) growth of *Aquaspirillum* spp. (3 x 10³ 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

<table>
<thead>
<tr>
<th>Phenotypic characteristic</th>
<th>Leptospira spp. strain lepto106</th>
<th>Aquaspirillum spp. strain aquas106</th>
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<tbody>
<tr>
<td>Growth at 13°C</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Growth at 28-30°C</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Growth at 4-8°C</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Growth at pH &lt;7.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5-fluorouracil resistance</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>Growth in EMJH medium</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Growth in Thioglycollate medium</td>
<td>-</td>
<td>X</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Helicoidally form</th>
<th>Flexuous helical cells</th>
<th>Rigid helical cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells dimensions</td>
<td>10-20 μm in length</td>
<td>10-60 μm in length</td>
</tr>
<tr>
<td></td>
<td>and less than 0.22 μm of diameter</td>
<td>and less than 0.22 μm of diameter</td>
</tr>
<tr>
<td>Motility</td>
<td>By axial filaments</td>
<td>By polar flagellum</td>
</tr>
</tbody>
</table>

### Incubation and growth at 28-30°C

a) Leptospires alone (*Leptospira* spp. strain lepto106): In liquid EMJH medium leptospires reached their maximum development (3 x 10³ 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 10⁵ 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 10⁵ 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 medium spirilla bacteria showed a progressive growth, reaching 3 x 10⁵ 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 10⁵ 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 10⁶ cells / ml (Figure 2B); then in death phase cell motility 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 10⁵ cells / ml to nearly 5 x 10⁶ cells / ml during the first three weeks. Approximately 2.5 x 10⁶ 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 10⁶ 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 10⁷ cells / ml in the first week up to week five of incubation. In this fluid leptospires growth similarly to that EMJH media (Figure 3C).

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**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 4-8°C

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 28-30° 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).
weeks. Spiral 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 spiral 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 strain aquas106 did not develop during three weeks. In semi-solid EMJH media, Leptospira spp. strain lepto106 showed more cell number / 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 spiral 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 spiral 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 & et. 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 spiral bacteria share similar habitats in nature, the presence of Aquaspirillum spp. in water would help to increase the average life of leptospirosis in the environment. All isolates were negative by Multiple-Locus Variable-number tandem repeats Analysis (MLVA), however, molecular identification by 16S rDNA gene sequences verified that all isolates were identified as non-pathogenic leptospires. Further studies will aim to serogroups circulating leptospires in surface waters.

REFERENCES

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

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<thead>
<tr>
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<tbody>
<tr>
<td>Liquid EMJH medium</td>
<td>168</td>
<td>84</td>
<td>77</td>
</tr>
<tr>
<td>Semi-solid EMJH medium</td>
<td>168</td>
<td>84</td>
<td>84</td>
</tr>
<tr>
<td>Stream water</td>
<td>112</td>
<td>98</td>
<td>56</td>
</tr>
</tbody>
</table>

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. Spiral 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 10^5 cells / ml, and in semi-solid EMJH medium to remain viable after 12 weeks.


