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Cell aggregation: a mechanism of pathogenic *Leptospira* to survive in fresh water

Summary. Transmission of leptospirosis is facilitated by the survival of pathogenic leptospires in moist environments outside their mammalian host. In the present study, the survival mechanisms of *Leptospira interrogans* serovar Canicola in aqueous conditions and lack of nutrients were investigated. In distilled water, leptospires were able to remain motile for 110 days (pH 7.2). However, when incubated in a semi-solid medium composed of distilled water and 0.5% purified agarose (pH 7.2), they survived 347 days. In this viscous environment, aggregates of live spirochetes were observed. Neither antibiotics (e.g. tetracycline and ampicillin) nor nutrients inhibited leptospiral aggregation. Immunoblot analysis suggested that cells incubated in water down-regulate the expression of LipL31, an inner-membrane protein, but retain expression of other membrane proteins. These studies provide insights into the mechanisms by which pathogenic *Leptospira* survives for prolonged periods of time in natural aqueous environments, a key stage in the leptospiral lifecycle. [**Int Microbiol** 2004; 7(1):35–39]

Key words: *Leptospira* · starvation · fresh-water survival · cellular aggregation · aqueous habitats

Introduction

The spirochete genus *Leptospira* comprises five saprophytic genomospecies and at least nine pathogenic genomospecies, affecting animals including humans [17]. Both pathogenic and saprophytic strains can be isolated from rivers and lakes. Leptospiral transmission occurs through direct contact with infected mammals [17] or exposure to urine-contaminated water [14,15,19,27]. Outbreaks are frequently associated with heavy rainfall, particularly when flooding results [1,14,15,27]. Under these conditions, leptospires are washed off the surface of urine-contaminated soil and collect in rivers and puddles [1]. Infection occurs when people come in contact with these waters. Outbreaks have also occurred among athletes practicing water sports in pristine waters of lakes or rivers [19]. Pathogenic leptospires survive in moist soil and fresh water for long periods of time, especially when the pH is slightly alkaline [13,17]. Under laboratory conditions, a strain of serovar Javanica was reported to survive in distilled water (pH 7.8) for 152 days [6].

Little is known about the mechanisms by which pathogenic leptospires persist in aqueous environments, outside the mammalian host. Saprophytic leptospires have been found to be one of the most common organisms in multi-bacterial biofilms of water pipes [25]. *Leptospira*, like other spirochetes, is well adapted to viscous environments, in which they show greater translational motility than any other

bacteria [7,12]. Moreover, other investigators have observed that *Leptospira* is attracted to viscous environments [20]. In this study, we investigated the effects of viscosity and salinity on leptospiral survival in fresh water.

Materials and methods

Bacterial strains and culture. *Leptospira interrogans* serovar Canicola A13 was kindly provided by Carol Bolin at the National Animal Disease Center (Ames, Iowa, USA). Bacterial cells were passaged weekly in EMJH medium [5].

Incubation in aqueous solutions and semisolid fluid. Leptospiral cells in logarithmic phase (1.5×10^8 cells ml^{-1}) were centrifuged, the supernatant thoroughly drained, and cells resuspended in the original volume (20 ml) of sterile distilled water (pH 7.2). The pH was adjusted with 1 mM sodium hydroxide. Leptospires were also incubated in solutions with varying concentrations of NaCl (0.13–0.85%), pH 7.2. Cells incubated in liquid media were counted weekly using a Petroff Hauser chamber (10 replicates). Alternatively, 2 ml cell suspension (from 20 ml of culture) was placed in 18 ml sterile semisolid fluid containing 0.2% agarose (molecular biology quality, Sigma, St. Louis, MO, USA), in distilled water, pH 7.2. The mixture was homogenized and poured into a Petri dish, which was placed in a sealed container and incubated in the dark at 20°C. To study long-term survival, cell suspensions obtained as previously described were placed in screw-capped tubes containing 10 ml of a semisolid media (0.5% agarose in distilled water, pH 7.2). Culturable cells were detected by inoculating 0.5 ml fluid in 7 ml semi-solid EMJH and incubating for 4 weeks. The typical gyrations in liquid media or corkscrew motility in agarose patches identified live leptospires. To test survival in other viscous environments, leptospires were incubated in a solution containing 2–4% methylcellulose (pH 7.2).

To differentiate leptospiral aggregation from growth, leptospires were incubated in semisolid fluid dishes containing either ampicillin (50 µg ml^{-1}), which kills dividing cells [21], or tetracycline (25 µg ml^{-1}), a bacteriostatic antibiotic. To test antibiotic inhibition of growth, bacterial cells were also placed in EMJH media and EMJH media plus antibiotic (ampicillin or tetracycline) and cultured for 2 weeks.

Chemotaxis assays. In order to determine the nature of the attractant causing cell aggregation, the procedure of Greenberg and Canale-Parola [8] was used. Briefly, U shaped tubes were placed onto a microscope slide and covered with a cover slip. The space was filled with leptospiral cell suspension containing approximately 1.4×10^8 cells ml^{-1}. Cells were suspended either in chemotaxis buffer (0.01 M potassium phosphate, pH 7.2) or distilled water (pH 7.2). Capillary tubes containing either 1 ml cell lysates or chemotaxis buffer were introduced into the chamber formed by the cover slip and the microscope slide. Capillary tubes were removed at 1, 2, and 4 h, and cells inside the capillary were counted using a Petroff Houser chamber. A second approach consisted of removing 20 ml of an exponential-phase culture (1.56×10^8 cells ml^{-1}) centrifuging it at 5,000×*g*, 25°C, 6 min, and resuspending the pellets in 2 ml sterile distilled water (pH 7.2). The cell suspension was placed in 18 ml molten 0.2% agarose in distilled water (pH 7.2, kept at 25°C), homogenized, and poured into a sterile Petri dish. Once the solution settled, 3 µl cells lysate was either placed over a 5-mm paper filter located on the surface of the agar or injected directly into the plate. Dishes were placed in sealed containers, incubated at 20°C for 2 weeks, and monitored daily for visible cell aggregation. Cell lysates were prepared from leptospiral cells grown to exponential phase in EMJH medium. Cells were centrifuged at 15,000×*g*, 5°C, 15 min, and washed twice with distilled water (pH 7.2). The pellet was resuspended in 5% of the original volume of distilled water, sonicated for 3 min on an ice bath, and frozen at -20°C until needed.

SDS-PAGE and immunoblot. Electrophoresis of proteins from whole leptospiral cell was carried out following a previously published protocol [16]. Each lane was loaded with 50 µl of a suspension containing 5×10^8 cells, either of water-incubated or EMJH medium-cultured leptospires. Immunoblot analysis was carried out as described [26].

Polyclonal antisera. Polyclonal antibodies to leptospiral GroEL were a generous gift of B. Adler (Monash University, Clayton, Victoria, Australia). Antisera to OmpL1 [10], LipL32 [9], LipL41 [23], ImpL63 [11], LipL31 [11], and LipL45/p31 [18] were prepared by immunizing New Zealand white rabbits with purified His6 fusion proteins as described in the respective publications.

Electron microscopy. Leptospires incubated in distilled water as described above were centrifuged at 5,000×*g*, 25°C, 6 min, and resuspended at a concentration of approximately 1×10^8 cells ml in Karnovsky's fixative (2% glutaraldehyde, 2.5% paraformaldehyde). Leptospires cultured in EMJH medium were washed twice in distilled water and fixed by the same procedure. Ten microliters of cell suspension was adsorbed to Formvar-coated 200-mesh copper grids (Ted Pella, Tustin, Calif., USA) for 1 min, after which the samples were stained with 1% uranyl acetate for 1 min. The grids were examined in a JEOL 100CX electron microscope at an accelerating voltage of 80 kV.

Results

Motile leptospires were observed for 98 days and were culturable for 110 days (data not shown) when incubated in distilled water (pH 7.2). *Leptospira* survived only 2 weeks in 0.85% NaCl (Fig. 1). Leptospiral motility in liquid media was constant and consisted of gyrations. Cell clumping or auto-agglutination due to increased stickiness of the cells was not observed.

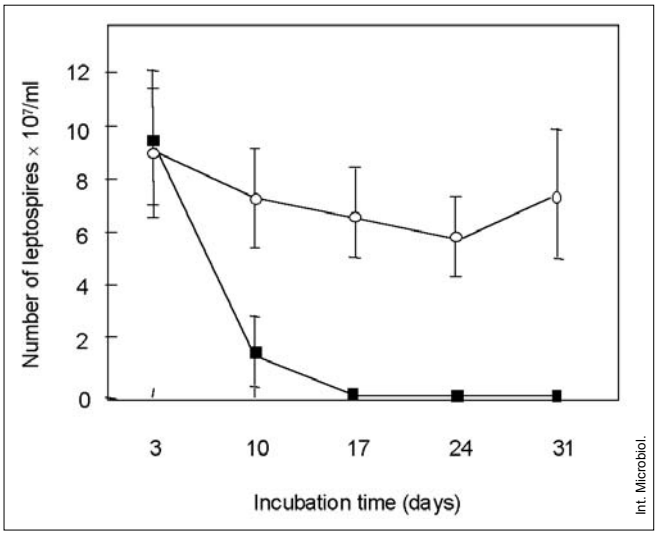

Fig. 1. Numbers of motile *Leptospira interrogans* cells as observed by dark-field microscopy. Cells were incubated either in distilled water (pH 7.2) (open circles) or 0.85% NaCl (closed squares). Vertical bars: standard deviation.

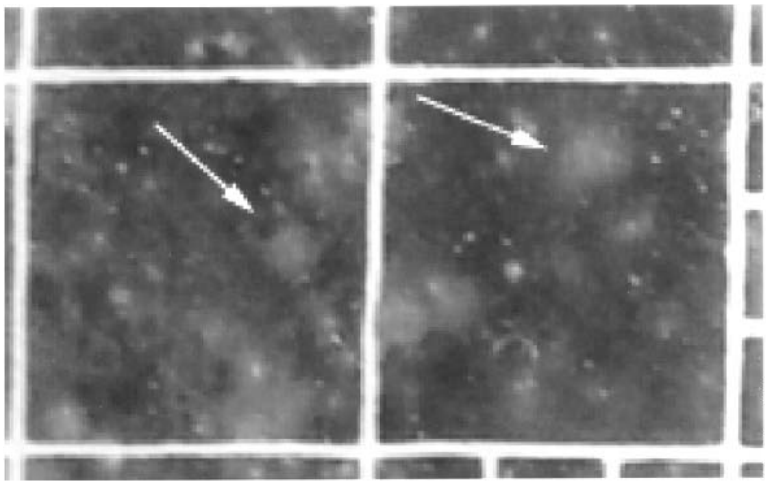

Fig. 2. Leptospiral cell aggregation. Arrows point to aggregates after 1 month incubation in 0.2% agarose (pH 7.2). Squares: 1 cm^2.

Incubation of *L. interrogans* in semi-solid fluid produced cell aggregations that became visible after 48 h as white spots in the translucent media (Fig. 2). These spots were dissected and analyzed by dark-field microscopy (Fig. 3). In this fluid, motile spirochetes were observed to form groups for up to 240 days, and remained culturable for 345 days. In semisolid fluid, bacterial cells showed only occasional corkscrew motility.

Leptospiral aggregation seemed to occur at the place of injection of the cell extracts, and where the surface of the agar was disrupted or dented. It was not possible to determine either the origin or the nature of the possible attractant responsible for the aggregation. Neither ampicillin nor tetracycline inhibited the aggregation of cells incubated in agarose solution. Both antibiotics inhibited bacterial growth when leptospires were placed in EMJH media. Leptospires incubated in methylcellulose solutions did not aggregate nor did they show increased survival.

Leptospires incubated in distilled water for a month showed abundant membrane blebbing and vesicle formation, as determined using electron microscopy (data not shown). It was impossible, however, to determine whether these changes were present in live or dead cells.

Protein profile analysis (SDS-PAGE) of cells incubated in water and EMJH medium did not show any differences. Immunoblot analysis, however, revealed an apparent reduction of expression of the protein LipL31, as determined by the reduced thickness of the protein band recognized by the corresponding antibody (Fig. 4).

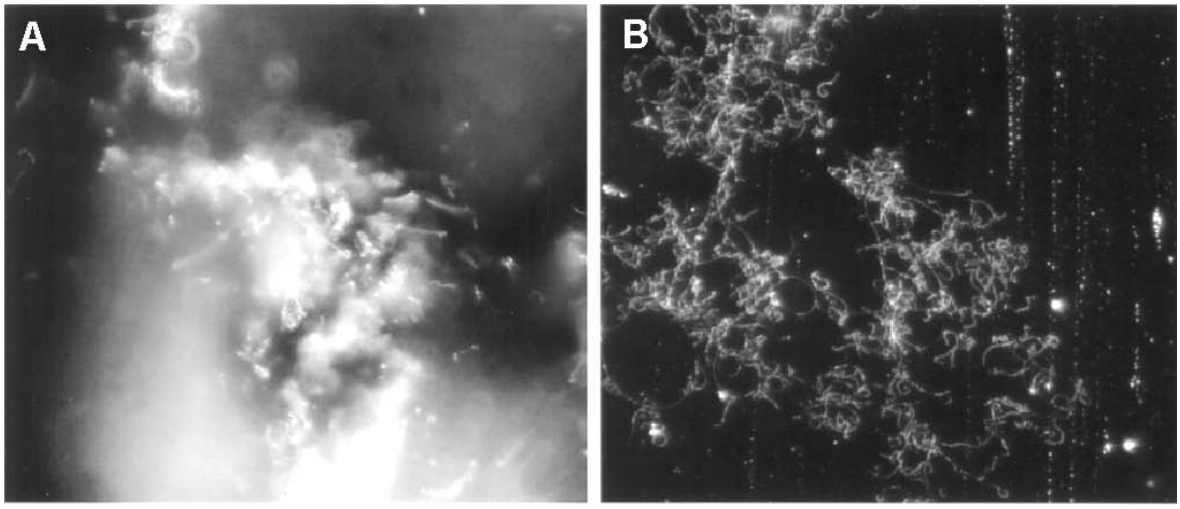

Fig. 3. Dark-field microscopy of aggregates of *Leptospira interrogans* serovar Canicola, incubated in a semisolid fluid (0.2% agarose, pH 7.2). **A**, 60-day incubation; **B**, 240-day incubation (400×).

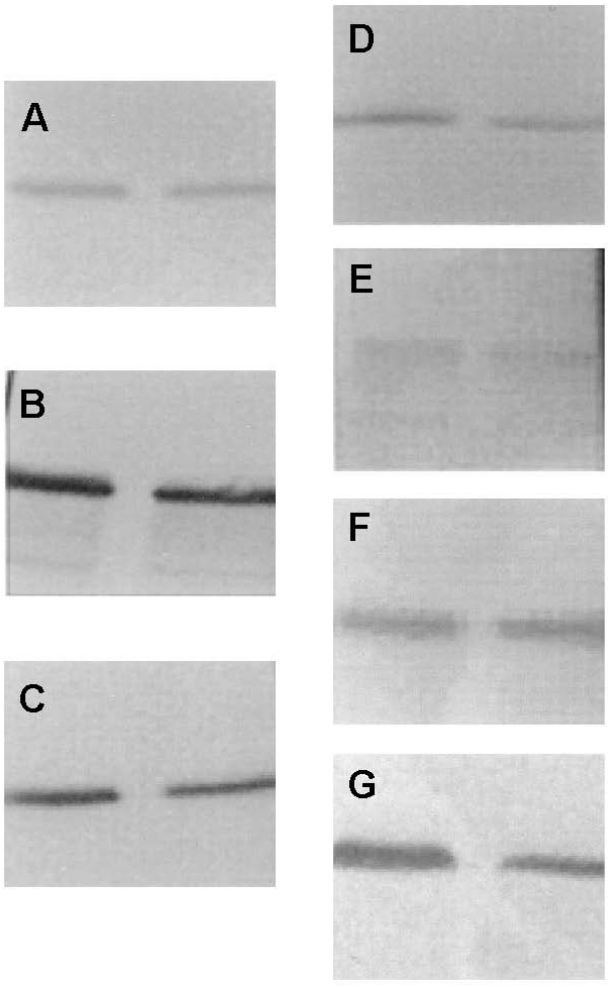

Fig. 4. Immunoblot analysis of leptospiral whole cell antigens, using rabbit antisera against: **A**, LipL41; **B**, leptospiral GroEL; **C**, LipL45; **D,** OmpL1; **E**, ImpL63; **F**, LipL32; **G**, LipL31. Left lanes: cells incubated for 1 month in EMJH medium. Right lanes: cells incubated for 1 month in distilled water (pH 7.2). Each lane received 50 µl of a suspension containing 5×108 cells per ml. Incubations were carried out at 20°C.

Discussion

The results presented here reveal some of the factors and mechanisms involved in the survival of pathogenic *Leptospira* in fresh water under low-nutrient conditions. Previous reports have identified pH as an important factor for leptospiral survival in fresh water [6]. The data here suggest that viscosity and salt concentration are also critical.

Leptospiral cells survived in distilled water for 110 days. When incubated in viscous solutions, however, the survival time of *Leptospira* increased more than three-fold (347 days). Microscopy of these viscous solutions showed aggregates of live leptospires with a few free-swimming spirochetes. Antibiotics did not affect aggregation, which indicated that leptospiral clusters were not the result of cryptic growth. This aggregation seemed to be the result of chemotactic attraction of leptospires to each other rather than increased cell stickiness. We were not able to determine the origin or chemical nature of the putative attractant. Unlike in other bacteria [2,4,24], leptospiral cell aggregation did not seem to be affected by the presence of nutrients.

Viscosity may favor leptospiral cell aggregation in two ways: (1) it provides a matrix that keeps cells together; (2) it facilitates spirochetal translational (directional) motility, and consequently chemotaxis [7,12]. Cell aggregation may help bacteria to endure harsh conditions by accumulating enzymes and nutrients from lysing cells [2,4]. Alternatively, it is possible that agarose binds toxic products and therefore increases the possibility of leptospiral survival. To examine this hypothesis, cells were incubated in viscous solutions containing methylcellulose. Substitution of methylcellulose for agarose did not reproduce the effects seen with agarose. Neither cell aggregation nor increased survival was observed (data not shown). Leptospiral cells incubated in water or liquid media showed constant gyrations, whereas motility was occasional in semi-solid media. Therefore, it is possible that cells in agarose used less energy than cells in liquid environments.

As a pathogen, *Leptospira* was expected to survive longer in isotonic saline solutions than in water. However, leptospiral survival was affected even at a NaCl concentration of 0.13% (data not shown). These results confirm that salts could be inhibitory for pathogenic *Leptospira* under starvation conditions, which has been suggested previously [3,22]. Pathogenic leptospires may require energy to survive in a medium that contains salts.

The immunoblot analysis of water-incubated cells showed an apparent down-regulation of the inner membrane protein LipL31. This result indicates that specific changes in the leptospiral-membrane protein profile are involved in the response to nutrient-poor and low-osmotic conditions.

Our study may provide evidence of social behavior in spirochetes [2,4,24], which phenomenon 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. Cell aggregation may also be responsible for the prolonged survival times observed when leptospires are cultured in semi-solid media [28].

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Agregación celular: un mecanismo de especies patógenas de *Leptospira* para sobrevivir en agua dulce

Resumen. La supervivencia de *Leptospira* en ambientes húmedos, fuera del mamífero hospedador, facilita la transmisión de la enfermedad. Nosotros estudiamos la supervivencia de *Leptospira interrogans* serovar Canicola en medios acuosos carentes de nutrientes. En agua destilada (pH 7,2), las leptospiras conservaron la movilidad durante 110 días. Sin embargo, cuando se incubaron en un medio semisólido, compuesto de agua destilada y 0,5% de agarosa purificada, sobrevivieron 347 días. En este medio viscoso se observó que las leptospiras formaban agregados. Ni la presencia de nutrientes ni la de antibióticos (ampicilina o tetraciclina) inhibieron la agregación. El análisis por inmunotransferencia indica que en las leptospiras incubadas en agua disminuye la expresión de la proteína de membrana interna LipL31; no obstante, conservan la expresión de otras proteínas de mem-

Agregação celular: um mecanismo de espécies patógenas de *Leptospira* para sobreviver em água doce

Resumo. A sobrevivência de *Leptospira* em ambientes úmidos, fora do mamífero hospedeiro, facilita a transmissão da enfermidade. Foi estudada a sobrevivência de *Leptospira interrogans* sorovar Canicola em meios aquosos carentes de nutrientes. As leptospiras em água destilada (pH 7,2) conservaram a mobilidade durante 110 dias. Quando foram incubadas em um meio semisólido, composto de água destilada adicionada de 0,5% de agarose purificado, as leptospiras sobreviveram 347 dias. Neste meio viscoso se observou que as leptospiras formam agregados. Nem a presença de nutrientes nem a de antibióticos (ampicilina ou tetraciclina) inibiu a agregação. A análise por imunotransferência indica que nas leptospiras incubadas em água há uma diminuição na expressão da proteína da membrana interna Lip L31, apesar de se conservar a expressão de outras proteínas da membrana. Este

brana. Este estudio proporciona una visión de los mecanismos que permiten la supervivencia de las especies patógenas de *Leptospira* en ambientes acuáticos naturales, un proceso importante en el ciclo natural de la leptospirosis. [**Int Microbiol** 2004; 7(1):35–39]

Palabras clave: *Leptospira* · escasez de alimentos · supervivencia en agua dulce · agregación celular · hábitats acuáticos

estudo proporciona uma visão dos mecanismos que permitem a sobrevivência das espécies patógenas de *Leptospira* em ambientes aquosos naturais, um processo importante no ciclo natural da leptospirose. [**Int Microbiol** 2004; 7(1):35–39]

Palavras chave: *Leptospira* · escassez de alimentos · sobrevivência na água doce · agregação celular · habitats aquosos