

Generation of Mammalian Host-adapted *Leptospira interrogans* by Cultivation in Peritoneal Dialysis Membrane Chamber Implantation in Rats

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[Abstract] *Leptospira interrogans* can infect a myriad of mammalian hosts, including humans (Bharti *et al.*, 2003; Ko *et al.*, 2009). Following acquisition by a suitable host, leptospires disseminate *via* the bloodstream to multiple tissues, including the kidneys, where they adhere to and colonize the proximal convoluted renal tubules (Athanzio *et al.*, 2008). Infected hosts shed large number of spirochetes in their urine and the leptospires can survive in different environmental conditions before transmission to another host. Differential gene expression by *Leptospira* spp. permits adaption to these new conditions. Here we describe a protocol for the cultivation of *Leptospira interrogans* within Dialysis Membrane Chambers (DMCs) implanted into the peritoneal cavities of Sprague-Dawley rats (Caimano *et al.*, 2014). This technique was originally developed to study mammalian adaption by the Lyme disease spirochete, *Borrelia burgdorferi* (Akins *et al.*, 1998; Caimano, 2005). The small pore size (8,000 MWCO) of the dialysis membrane tubing used for this procedure permits access to host nutrients but excludes host antibodies and immune effector cells. Given the physiological and environmental similarities between DMCs and the proximal convoluted renal tubule, we reasoned that the DMC model would be suitable for studying *in vivo* gene expression by *L. interrogans*. In a 20 to 30 min procedure, DMCs containing virulent leptospires are surgically-implanted into the rat peritoneal cavity. Nine to 11 days post-implantation, DMCs are explanted and organisms recovered. Typically, a single DMC yields ~10⁹ mammalian host-adapted leptospires (Caimano *et al.*, 2014). In addition to providing a facile system for studying the transcriptional and physiologic changes pathogenic *L. interrogans* undergo within the mammal, the DMC model also provides a rationale basis for selecting new targets for mutagenesis and the identification of novel virulence determinants.

Caution: Leptospira interrogans is a BSL-2 level pathogen and known to be excreted in the urine of infected animals. Animals should be handled and disposed of using recommended Animal Biosafety Levels (ABSL) for infectious agents using vertebrate animal guidelines.

Note: All protocols using live animals must conform to governmental regulations regarding the care and use of laboratory animals. The success of this protocol is dependent on the proper

use of aseptic techniques during all stages of both dialysis membrane chamber preparation and animal surgery.

Materials and Reagents

1. Adult female Sprague-Dawley rats (175-200 g)
2. EMJH medium (prepared as described in Supplementary data)
3. Bovine serum albumin (BSA)
EMJH+BSA (10 mg/ml BSA, BSA concentration 20 mg/ml) (Millipore, catalog number: 810037)
Note: The quality of the albumin used for the cultivation of leptospires is critical. Please verify that the albumin used for DMC implantation supports the growth of virulent leptospires under standard in vitro growth conditions. Other sources of BSA were tested (e.g., Millipore, catalog number: 840644 and Sigma-Aldrich, catalog number: A-9647) with similar results.
4. *Leptospira interrogans*, logarithmic phase, grown under standard *in vitro* conditions (28-30 °C) (Zuerner, 2005)
5. Ultrapure water (deionized, distilled) (e.g., Milli-Q)
6. 1 mM EDTA (pH 8.0)
7. Regenerated cellulose dialysis membrane tubing (Spectrum™ Spectra/Por™ 1 RC, 6,000 to 8,000 MWCO, 32 mm width) (Spectrum Labs, catalog number: 132655)
8. Filter units (0.22 µm, 250 ml, sterile) (e.g., Millipore Stericup® filter unit)
9. Ketamine/xylazine anesthetic cocktail (40-80 mg per kg/ 5-10 mg per kg, administered intramuscularly)
10. Ophthalmic ointment (e.g., Puralube)
11. Betadine® surgical scrub solution
12. Carprofen (5-10 mg per kg, administered subcutaneously)
13. Ethanol (70%)
14. Ketamine/xylazine anesthetic cocktail (see Recipes)
15. EMJH + BSA medium (see Recipes)

Equipment

1. Surgical gloves (individually wrapped, sterile)
2. Disposable serological pipets (10 ml sterile, individually-wrapped)
3. Surgical drape (cut into 12 in (46 cm) squares, sterile)
4. Gauze (4 x 4 in sterile)
5. Microscope equipped with a dark field condenser (e.g., Olympus, model: BX40)
6. Three 2-L Pyrex beakers (each containing a magnetic stir bar)
7. Hot plate with stirring option

8. Extra-long blunt end forceps (sterile)
9. Pipet filler (e.g., Drummond Scientific, model: Pipet-Aid)
10. Surgical instrument pack, one per animal, sterilized prior to use and kept within sterile package:
 - Scalpel blades (No. 10)
 - Scalpel blade holder
 - Scissors, iris, 4 in (~10.2-cm)
 - Tissue forceps, 5 ½ in (~14 cm) (1x 2-tooth dissecting or Adson-Brown)
 - Tissue forceps, 5 ½ in (~14 cm) (blunt-end)
 - Needle holder forceps with built-in scissors (e.g., Olsen Hegar) (5 ½ in (~14 cm))
 - Suture, Ethicon 40, SH 1, 27 in (~68.6 cm) coated Vicryl, violet-braided suture
 - Autoclip® 9-mm stainless steel wound closure clips and applicator
11. Biological safety cabinet (Biosafety Level 2; BSL2)
12. Circulating warm-water blanket and pump
13. Electric hair/fur clippers
14. Glass bead sterilizer (e.g., Braintree Scientific, model: Germinator 500) (optional)

Procedure

A. Preparation of sterile dialysis membrane tubing

1. Fill three 2-Liter Pyrex beakers as follows:
 - Beaker 1: 1 - 1.5 L Ultrapure water
 - Beaker 2: 1 - 1.5 L 1 mM EDTA (pH 8.0)
 - Beaker 3: 1 - 1.5 L Ultrapure water
2. Add a magnetic stir bar to each beaker, cover with heavy-duty aluminum foil and autoclave.

Note: To ensure sterility, beakers should be autoclaved on the same day at the procedure.
3. Place each beaker on hot plate and bring to a rolling boil with constant stirring.
4. Wearing sterile gloves, cut dialysis membrane tubing into strips 7-9 inches (~18-23 cm) in length using sterile scissors. Cut one strip per animal plus 1-2 extra.
5. Gently tie off one end of the tubing using a simple overhand knot by forming a loop at the top of the DMC and passing the free end of the tubing through the loop. Trim away excess tubing from the tied end using sterile scissors.
6. Place the tied tubing into Beaker 1 and replace the aluminum foil cover. Be sure to keep the foil cover loose enough to allow steam to escape. Boil tubing for 20 min. with constant stirring.

Note: Tubing should remain submerged at all times.

7. Using sterile extra-long forceps, transfer tubing to Beaker 2. Replace aluminum foil cover and boil tubing for 20 min. with constant stirring.
8. Repeat step A7 using Beaker 3.
9. Once cool to the touch, transfer Beaker 3 to a Biological safety cabinet (BSC). To ensure sterility, the beaker containing the tubing should remain in the BSC.

Note: Dialysis membrane tubing may be prepared several days in advance and stored at 4 °C. After boiling, transfer dialysis membrane tubing to the bottom portion of a 0.22 µm Stericup® Filter Unit (Millipore) using sterile blunt tip forceps. Replace the filter unit top and filter in ~100-200 ml of Ultrapure-Q water from Beaker 3. Seal the bottom portion of the filter unit using the sterile cap provided by the manufacturer. The container should be opened only within the BSC.

B. Preparation of leptospire

1. Use a standard low passage *in vitro* culture of virulent *L. interrogans*, kept at 28-30 °C in EMJH medium, before it reaches stationary phase in the growth curve.
2. Just prior to starting the surgical procedure, count bacteria by dark field microscopy.
3. Dilute culture to 10⁶ organisms/ml using fresh EMJH medium.
4. Working in the BSC, transfer 10 ml of EMJH+BSA to sterile 50-ml conical tubes. Prepare one conical tube per DMC.

Note: It is important that the EMJH medium used for DMCs is supplemented with additional BSA to maintain the appropriate osmotic pressure.

5. Add 0.1 ml of dilute culture to each 50-ml conical tube containing EMJH+BSA medium (Final density, 10⁴ organisms/ml).

C. Preparation of DMCs

1. Working in the BSC, place several disposable, individually-wrapped serological pipets within the surgical field. Lay down a sterile drape to use as a workspace. Clean the surface of an automatic pipeter using 70% ethanol and place on the sterile drape. After donning a new pair of sterile surgical gloves, loosen the cap of a 50-ml conical tube.
2. While still wearing gloves, carefully remove a strip of tubing from its storage container using blunt end forceps. Use your free hand to hold the tubing between index finger and thumb. With other hand, remove the cap from the 50-ml conical tube containing diluted bacteria and transfer up to 9 mls to the tubing using a 10-ml disposable serological pipette. While continuing to hold the open end, flatten the unfilled tubing between the fingers of your free hand to eliminate any air bubbles.
3. Gently twist the top end of the tubing (1-2 turns) to remove any remaining void volume and close off the top of the DMC Use your thumb and index finger to hold on to the twisted area to prevent liquid from leaking out of the DMC while you tie off the top. Tie off the open end using a simple overhand knot (*e.g.*, form a loop at the top of the DMC, passing the free end of the tubing through the loop, then, without releasing your thumb

and index finger, gently tighten the knot). Trim away excess tubing from the tied end using sterile scissors.

Note: Try to make the DMC as taut as possible; this will help to maneuver the chamber into the peritoneal cavity during surgery. The resulting filled DMC should be ~1.5 - 2 in. Larger DMCs may interfere with normal intestinal or bladder functions.

4. Place the filled DMC into a sterile 50-ml conical tube containing ~5-7 ml of fresh EMJH+ BSA medium.
5. Repeat steps C3-4 until all bags are filled then proceed directly to the surgical procedure.

Note: It is not recommended that filled dialysis membrane chambers be stored for extended periods of time as this may increase the risk of contamination.

D. Peritoneal implantation procedure.

1. Anesthetize animal by intramuscular injection with the ketamine/xylazine cocktail.
2. Apply a small amount of ophthalmic ointment to each eye.
3. Administer preoperative analgesia (e.g., Carprofen).
4. Shave the abdomen and prepare surgical site (e.g., successive washes with Betadine® Surgical solution followed by alcohol).
5. Cover the surgical site with sterile gauze and transfer the animal to the BSC in a supine position.
6. Prior to beginning the procedure, remove the sterile gauze covering the surgical site and perform a 'toe pinch' to ensure that the animal is properly anesthetized.
7. Using a sterile scalpel blade, make a 5 cm incision through the skin only, starting ~2.5 cm below the ribcage, using the xiphoid process as a guide. Using tissue forceps, pull up the skin on either side of the incision and gently trim the fascia connecting the skin to the abdominal wall using a scalpel. Repeat on the other side.
8. Use the same scalpel, make a small incision (~4 cm) in the abdominal wall, using the *linea alba* as guide. The incision should be clean and straight.
9. Using tissue forceps, raise one side of the abdominal incision and place a DMC inside rat peritoneal cavity (Figure 1). Gently push the DMC towards either side of the peritoneal cavity to prevent it from being nicked/ruptured during suturing. Be sure to position so that it does not become entangled in the intestines or interfere with bladder expansion.



Figure 1. Implantation of DMC into rat peritoneal cavity

10. Close the abdominal incision site by suturing. Begin by suturing each end of the incision with double knots. Working upwards, close the remaining incision by placing sutures ~2-3 mm apart.
11. Close the skin incision site using a contiguous line of AutoClip® wound clips (Figure 2).
Note: Alternatively, the skin excision site may be closed using subcuticular stitches followed by liquid skin adhesive (e.g., Nexaband).



Figure 2. Closing the skin incision

12. Place the rat on top of a clean surgical drape in a clean cage containing fresh bedding. Place the cage on top of a circulating water heating pad to maintain the appropriate core body temperature. Monitor the animal continuously until alert and responsive.
 13. Analgesia should be administered for at least two days post-operatively.
 14. Animals should be monitored at least once daily for the first week post-operatively and then every other day thereafter. If animals show any sign of distress or discomfort, consult institutional veterinary staff immediately.
- E. Recovery of mammalian host adapted organisms from DMCs
1. At 9 to 12 days after implantation, euthanize animals by CO₂ asphyxiation.
 2. Place animal in a BSC in a supine position.
 3. Using sterile surgical scissors, expose the abdominal wall.

4. Using sterile tissue forceps, lift one side of the sutured abdominal incision site and re-open using sterile scissors.
5. Locate and remove the DMC using blunt-end forceps. Transfer to a sterile 50-ml conical tube.
6. Using sterile forceps to hold the DMC by one knot, make a small cut in the tubing just below the knot. Slowly remove the contents of the DMC using a sterile disposable serological pipet. Transfer the DMC fluid to a sterile 15-ml conical tube. Alternatively, DMC contents can be removed using a syringe 18-G, 1 in. needle attached to a sterile 10-ml syringe. However, aspiration of leptospires by syringe may disrupt cells and/or cause cell lysis.
7. Examine a small aliquot of the contents DMC fluid from each chamber under dark field microscopy.
8. Proceed to downstream applications (e.g., RNA isolation, analysis by 1D and 2D SDS-PAGE).

Representative data

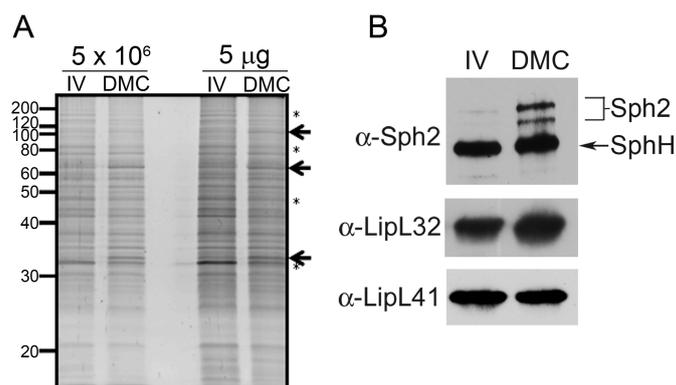


Figure 3. Virulent leptospires become mammalian host-adapted during growth within dialysis membrane chambers. Representative whole cell lysates of leptospires cultivated to late-logarithmic phase in EMJH medium at 30 °C *in vitro* (IV) and within dialysis membrane chambers (DMC) implanted into the peritoneal cavities of female Sprague-Dawley rats. A. Lysates were loaded according to the numbers of leptospires (5×10^6 per lane) or total protein (5 μ g per lane) and stained with SYPRO Ruby gel stain. Arrows and asterisks are used to highlight examples of polypeptides whose expression appears to be increased or decreased, respectively, within DMCs compared to *in vitro*. Molecular mass markers are indicated on the left. B. Immunoblot analyses using rabbit polyclonal antisera directed against Sph2 (Matsunaga *et al.*, 2007), LipL32 (Haake *et al.*, 2000) and LipL41 (Shang *et al.*, 1996). An arrow is used to indicate a band of the predicted molecular mass for SphH, a second, closely related sphingomyelinase in *L. interrogans* recognized by antiserum directed against Sph2 (Matsunaga *et al.*, 2007; Carvalho *et al.*, 2010). Image reproduced from Caimano *et al.* (2014).

Recipes

1. Ketamine/xylazine anesthetic cocktail

Stock solutions

100 mg/ml ketamine-HCl

20 mg/ml xylazine-HCl

2. EMJH + BSA medium

Prepare 1-L of EMJH medium as described in Supplementary data and previously published (Zuerner, 2005)

Weight out 10 mg of BSA using an analytical balance

Slowly stir in 10 mg of BSA with constant stirring, avoiding bubbles, until the BSA is completely dissolved

Sterilize by filtration using 0.22 µm Stericup® Filter Unit

Stored at 4 °C

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