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A mouse model of food borne *Listeria monocytogenes* infection

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Abstract

Listeria monocytogenes cause foodborne disease in humans that ranges in severity from mild, self-limiting gastroenteritis to life-threatening systemic infections of the blood, brain, or placenta. The most commonly used animal model of listeriosis is intravenous infection of mice. This systemic model is highly reproducible, and thus, useful for studying cell-mediated immune responses against an intracellular bacterial pathogen, but it completely bypasses the gastrointestinal phase of *L. monocytogenes* infection. Intragastric inoculation of *L. monocytogenes* produces more variable results and may cause direct bloodstream invasion in some animals. The food borne transmission model described here does not require specialized skills to perform and results in infections that more closely mimic human disease. This natural feeding model can be used to study both the host and pathogen-derived factors that govern susceptibility or resistance to orally acquired *L. monocytogenes*.

Keywords

listeriosis; oral transmission; intestines; intracellular pathogen

INTRODUCTION

Listeria monocytogenes cause gastroenteritis and then spread systemically in a subset of people who ingest contaminated food, resulting in septicemia and meningoencephalitis with high mortality, despite antibiotic treatment (Allerberger et al., 2010). Oral transmission of *L. monocytogenes* is relatively inefficient in mice, and the most commonly used model of oral transmission has been to intragastrically (i.g.) inject high doses of bacteria (10^9–10^{11}CFU) suspended in PBS directly into the stomach. Typically, this results in rapid bacterial transit through the gastrointestinal tract (with much of the inoculum shed in feces), and very rapid systemic spread, essentially creating a lethal intravenous (i.v.) infection. This does not closely mimic human disease, where delays of up to 3 weeks can occur before systemic symptoms appear (Gellin and Broome, 1989).

In Basic Protocol 1, mice are fed *L. monocytogenes*-contaminated pieces of bread (see Support Protocol 1). The total number of *L. monocytogenes* shed in the feces (see Basic

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Protocol 2) and the number of either luminal or cell-associated *L. monocytogenes* in each tissue of the gastrointestinal tract (see Basic Protocol 3) can be determined using selective agar media (see Support Protocol 2). If desired, the infected gut tissues can be fractionated, allowing for enumeration of total bacteria in the mucus layer, and both intracellular and extracellular *L. monocytogenes* found in either intestinal epithelial cells or the cells of the underlying lamina propria (see Alternate Protocol). After a reproducible delay, *L. monocytogenes* begin to disseminate, first to the mesenteric lymph nodes (MLN) and then to peripheral tissues. The total number of bacteria found in the MLN, spleen, liver, gall bladder, and brain can then be determined (see Basic Protocol 4).

NOTE: Listeria monocytogenes is a Biosafety Level 2 (BSL-2) pathogen. All procedures must be performed following the appropriate guidelines for handling pathogenic microbes. See *UNIT 1A.1* and other pertinent resources (*APPENDIX B*) for more information.

NOTE: This protocol requires Animal Biosafety Level 2 (ABSL-2) conditions. Follow all appropriate guidelines for the use and handling of infected animals. See *UNIT 1A.1* and other pertinent resources (*APPENDIX B*) for more information. All experiments using mice must be pre-approved by an Institutional Animal Care and Use Committee (IACUC).

BASIC PROTOCOL 1 INFECTION OF MICE BY THE NATURAL FEEDING ROUTE

In this oral transmission model of listeriosis, mice ingest small pieces of bread contaminated with *L. monocytogenes* (see Support Protocol 1). This infection route, used at doses of 10^7–10^9 CFU, results in transient intestinal colonization of resistant C57BL/6/J mice and prolonged infection of susceptible BALB/c/By/J mice (Bou Ghanem et al., 2012). The total number of *L. monocytogenes* shed in the feces of individual mice can be monitored over the entire course of the infection (see Basic Protocol 2). In addition, the bacterial loads in the gut tissues (see Basic Protocol 3) and the number of *L. monocytogenes* that disseminate to peripheral tissues (see Basic Protocol 4) can be determined at various time points post-infection.

Materials

6- to 9-week old mice

Microisolator cage bottoms, empty

Raised (1 inch) wire flooring units (#3 mesh; Allentown)

L. monocytogenes-contaminated bread pieces *(see Support Protocol 1)*

Tissue forceps, sterile

1. Fast mice one day (~18–24 h) prior to infection by removing the food and placing a raised wire flooring unit in the cage to prevent coprophagy. Allow unrestricted access to water.

   Leave only a minimal amount of bedding in the cage to absorb urine. If the bedding material is too high, the mice will be able to reach excreted

feces, pick it up and eat it. Do not add cotton squares to the cage for enrichment.

2. Place a single mouse in a completely empty (no bedding) microisolator cage. Using sterile forceps, transfer one contaminated bread piece to the bottom of the empty cage.

   Typically, the mouse will pick up the bread piece and consume it entirely within 1–2 min. If the mouse does not eat the bread right away, cover the cage and leave the mouse undisturbed for up to 2 h, and then check for the absence of the bread piece. Some mouse strains are more receptive to feeding during their dark cycle (see Commentary).

3. Return the mouse to its original cage and replenish the mouse chow. Continue to house the mice on raised wire flooring for the duration of the experiment.

BASIC PROTOCOL 2 MONITORING THE LEVEL OF *L. MONOCYTOGENES* SHED IN FECES

It has been estimated that 90% of an oral L. monocytogenes inoculum is killed during exposure to the harsh environment (low pH and high osmolarity) of the upper GI tract (Sleator, R.D. et al., 2009). For the bacteria that survive passage through the stomach, a significant portion of either ingested (Bou Ghanem, et al., 2012) or i.g. inoculated (Hardy, et al., 2004) *L. monocytogenes* are quickly shed in the feces within 3 hours of infection. Once intestinal colonization has been established, susceptible animals will continue to shed high levels of *L. monocytogenes* for up to two weeks. Thus, the amount of *L. monocytogenes* present in excreted stool can be used as a non-invasive marker for the level of intestinal infection in mice.

Materials

Infected 6–9 week old mice (Basic Protocol 1)

500 ml beakers, sterile

1.5 ml microcentrifuge tubes, sterile

Tissue forceps, sterile

Toothpicks, sterile

Phosphate buffered saline (PBS), sterile

BHI/L+G agar plates (see Support Protocol 2)

Microcentrifuge

Vortex

37°C incubator

1. Label one microcentrifuge tube for each mouse that will provide a stool sample. Weigh the tube and record the weight in milligrams.

2. Open a cage, and quickly place each mouse in a separate 500 mL beaker. Wait until the mouse excretes at least one fecal pellet, and then return each mouse to the cage.

   It is important to work quickly after handling the cage, otherwise the mice will excrete all available feces while still in the cage, making it difficult to recover individual stool samples. Typically, the mice will expel 1–4 stool pellets within 2–5 minutes.

3. Use sterile forceps to transfer fecal pellets to the appropriately labeled microcentrifuge tubes.

4. Weigh each sample tube again. Calculate the fecal weight by subtracting the weight of the empty tube.

   Typical weights for stool samples range from 10–30 mg per pellet.

5. Add a standardized volume of PBS to each sample (100μl per 15 mg feces) and use a sterile toothpick to mash the fecal pellets.

6. Vortex each tube for 30 seconds, then prepare serial dilutions in sterile water or PBS and plate on BHI/L+G agar (see Support Protocol 2). Incubate at 37°C.

   The choice of dilutions to plate depends on both the bacterial isolate and the mouse strain being used, as well as the time point post-infection. BHI/L+G agar will inhibit the growth of most intestinal microbiota.

7. After 24–48 h incubation, count the number of colonies with a dome-shaped creamy white colony appearance and calculate the CFU/mg of feces.

   Suspect colonies can be verified by plating on Listeria ChromAgar. (Gibson Laboratories, LLC, Lexington, KY)

BASIC PROTOCOL 3 DETERMINATION OF BACTERIAL LOADS IN GUT TISSUES

This protocol describes the procedures for harvesting the stomach, duodenum, jejunum, ileum, cecum, colon, and the mesenteric lymph nodes (MLN) from infected mice and determining the total bacterial loads in each of these tissues at various time points post-infection. The contents of each intestinal tissue are flushed out and can either be discarded or processed for CFU determination (luminal bacteria). Then the total number of cell-associated (adherent extracellular plus intracellular) bacteria in the remaining tissue is determined. The draining lymph nodes for the gut (MLN) are processed separately.

Materials

Infected 6- to 9-week old mice (Basic Protocol 1)

70% ethanol in a spray bottle

Sterile dissection instruments including:

Medium dissection scissors

Tissue forceps

Small surgical scissors

Styrofoam or cork dissection board and dissecting pins

60 mm petri dishes, sterile

100 mm treated petri dishes, sterile

Phosphate-buffered saline (PBS), sterile

10 ml slip tip syringes, sterile

25 g needles, sterile

500 ml glass beaker

polypropylene centrifuge tubes, labeled and filled with 2 ml of sterile water

Tissue Homogenizer (PowerGen1000 or similar)

50 ml polypropylene centrifuge tubes, sterile, filled with ~ 25 ml sterile H_2O

50 ml polypropylene centrifuge tubes, sterile, filled with ~ 25 ml 70% ethanol

1.5 ml microcentrifuge tubes, sterile, filled with 450 μl of sterile H_2O

stainless steel #80 mesh screens, sterile

3 ml syringes, sterile

BHI/L+G agar plates (see Support Protocol 2)

37°C incubator

Harvest of gut tissues

1 Euthanize the infected mouse and thoroughly spray the abdomen with 70% ethanol to disinfect and wet the fur.

If using multiple groups of animals, it is most efficient to euthanize all of the mice (up to 4 animals) in the experimental group at once.

2 Pin the limbs of each mouse to a dissecting board. Grab the skin with a pair of forceps, and then use medium dissection scissors to make a midline incision in the skin along the length of the mouse. Peel back the skin to expose the peritoneum.

3 Using small surgical scissors, cut through the peritoneal wall to expose the intestinal tissues.

Be careful not to nick the intestines, as this will make it more difficult to flush out the contents in step #9.

4 Remove the mesenteric lymph nodes (MLN), place in a 60 mm dish on ice, and remove all attached fat.

A lymph node will roll along the dish when dragged by a forceps with gentle pressure. Fat globules have a similar appearance, but will break

apart when gentle pressure is applied. Expect to find 3–5 nodes per mouse; the MLN will be enlarged in infected mice compared to uninfected mice.

5 Grab the middle of the stomach with a pair of forceps, and detach by making a single cut at the top where the stomach joins to the esophagus.

6 Gently pull on the stomach, drawing the intestines out of the peritoneal cavity. Holding the stomach end high above the carcass, detach at the lower end by making a single cut ~ 1 cm above the rectum. Transfer entire tissue to an appropriately labeled empty 100 mm petri dish.

Drawing out the entire intestinal tract by pulling gently will cleanly separate the intestines from the surrounding connective tissue and fat.

Flushing of luminal contents

7 Using small surgical scissors, cut the intestinal tissue to isolate the stomach, small intestines, cecum, and colon. Place each tissue to be analyzed in separate 60 mm dishes.

The small intestines can be further divided by length into three equal pieces approximating the duodenum, jejunum, and ileum. This is recommended to facilitate removal of the luminal contents. After flushing, the three pieces can then be processed either together (for total CFU per small intestines) or separately (for CFU per duodenum, jejunum, and ileum).

8 Wet each tissue by applying ~0.5 ml of sterile PBS and store on ice.

Wetting the tissues keeps them pliable and will prevent tears during handling.

9 Fill a 10 ml syringe with 8 ml of sterile PBS, attach a 25 g needle, and set aside. Grab one end of a single intestinal tissue (stomach, cecum, colon, or one-third of the small intestine) with sterile forceps and hold the tissue over a waste beaker. Using a second pair of forceps, squeeze out the luminal contents.

If enumeration of luminal *L. monocytogenes* is desired, collect the contents and flushes (see step # 8) into a sterile centrifuge tube. Centrifuge the pooled contents plus washes for 20 min. at 12,000 × *g*. Aspirate the liquid and suspend the pellet in 0.5 – 1.0 ml of sterile water. Prepare serial dilutions and plate on BHI/L+G agar.

10 Insert the tip of the needle into the opening at the top of the tissue and with flush 4 ml of PBS. Use forceps to squeeze out the contents. Flip the tissue over and repeat on the other end, flushing with the remaining 4 ml of PBS.

Be careful not to push the needle out of the lumen of the small intestine and colon pieces. The PBS should slightly inflate these tissues, and the

interior of the tissue should become transparent as the fecal matter and mucus are squeezed out.

Tissue homogenization

11 To quantify the total number of cell-associated *Listeria*, open each washed tissue by cutting longitudinally with sterile scalpel blade, and then make several lateral cuts to slice the tissue into smaller fragments.

This step is essential to ensure that the long pieces of intestinal tissue do not encircle and then get trapped within the homogenizer probe.

12 Transfer the intestinal pieces to an appropriately labeled centrifuge tube containing 2 ml sterile water.

Standard 15 ml polypropylene centrifuge tubes with a conical bottom can be used for this purpose, however, polypropylene tubes with a rounded bottom may be easier to use with the tissue homogenizer.

13 Disinfect a tissue homogenizer by placing the probe in a 50 ml centrifuge tube containing ~25 ml of 70% ethanol. Homogenize at 60% power for 10–15 sec.

Wait for the ethanol to completely air dry, or place the probe in another 50 ml tube containing ~25 ml of sterile H_2O and process at 60% power for 10–15 sec. to dilute the ethanol.

14 Homogenize each minced intestinal tissue for 1 min. Clean the probe between tissue samples by homogenizing for 10–15 sec. in sterile water and disinfect with 70% ethanol between sample groups.

Use a separate water tube for each sample group and tissue type (e.g. colons from experimental group A animals).

Mesenteric lymph nodes (MLN)

15 To process the MLN, place a sterile wire mesh screen in a 60 mm petri dish containing 0.75 ml of sterile water, and then transfer the nodes to the top of the mesh screen.

16 Use the plunger from a sterile 3 mL syringe to mash the nodes through the mesh screen.

Be sure to push down on the screen to the bottom of the dish so the screen makes contact with the water in the dish to release the cells stuck to the bottom of the screen.

17 Pipet 0.75 mL of sterile water through the screen and then pipet up and down several times to rinse the screen with the total volume of 1.5 ml. Transfer cell suspension to a microcentrifuge tube, and vortex vigorously for 30 sec. to lyse the cells.

Enumeration of bacteria

18 Prepare serial 10-fold dilutions of each sample in sterile water (50 µl of sample into 450 µl of water). Spread 50 µl samples of appropriate dilutions onto one-third sections of a BHI/L+G agar plate. Incubate for 36–48 h at 37°C.

Tiny colonies will be visible after 24–30 hours of incubation, but it will be easier to distinguish *L. monocytogenes* colonies (creamy white, dome-shaped) from any intestinal microbiota that are not inhibited by the selective agar after 48 h. Some inhibition of listerial growth may be noted for undiluted intestinal tissue homogenates plated on BHI/L+G agar. Suspect colonies can be verified by plating on *Listeria* ChromAgar. *(Gibson Laboratories, Lexington, KY)*

19 Calculate the CFU/ml by multiplying the number of *L. monocytogenes* colonies observed by 20 (since only 50 µl was plated) times the dilution factor. The total CFU per tissue can be determined by multiplying the CFU/ml by the total volume of the sample.

ALTERNATE PROTOCOL FRACTIONATION OF INTESTINAL TISSUES

L. monocytogenes can invade intestinal epithelial cells and is rapidly trancytosed to the underlying lamina propria. These events can be visualized in foci of infection using immunofluorescent microscopy. However, it is difficult to quantify the total number of bacteria that have invaded the gut mucosa using microscopic techniques. The alternate protocol outlined below separates gut tissues (small or large intestine) into three fractions: the mucus layer, the intestinal epithelium, and the cells within the underlying lamina propria. An optional treatment of single cell suspensions with gentamicin, an antibiotic that does not penetrate mammalian cells at low concentrations, can also be used reveal the number of intracellular bacteria contained within each fraction.

Materials

Intestinal tissues, flushed & longitudinally cut (see Basic Protocol 3)

16 × 79 mm polypropylene tubes, sterile

6 mM N-acetylcysteine in PBS (NAC), (see recipe)

mesh filters, sterile or disposable cell strainers

50 ml centrifuge tubes, sterile

scalpel blades, sterile

RP5/EDTA/DTT, sterile (see recipe)

RP5/HEPES, sterile (see recipe)

RP5/$Gent_{25,}$ sterile (see recipe)

Phosphate buffered saline (PBS), sterile

digestion solution (see recipe)

1 cm magnetic stir bars, sterile

microcentrifuge tubes containing 450 µl of sterile H_2O

BHI/L+G agar (Support Protocol 2)

Isolation of mucus

1 For each intestinal tissue to be processed, fill three 16 × 79 mm polypropylene tubes with 3 mL of 6 mM N-acetylcysteine (NAC).

2 Place the flushed and longitudinally cut tissue in the first tube for 1–2 min, vigorously swirling every 20–30 sec. Using a sterile forceps, pick up the tissue and gently squeeze against the side of the tube to remove excess liquid. Repeat twice more using the remaining two tubes filled with NAC.

3 Remove the intestinal tissue and set aside for further processing. Pool the NAC washes (total of 9 mL), and centrifuge for 20 min at 12,000 × *g*.

4 Re-suspend the pellet in sterile water and vortex for 30 sec. Store on ice until ready to dilute and plate.

Isolation of epithelial cells (EC)

5 Cut each tissue into small pieces with a sterile scalpel blade and place in a 50 mL tube containing 4 mL of RP-5/EDTA/DTT and incubate shaking (250 rpm) at 37°C for 20 min.

If desired, the visible Peyer's Patches can be removed from the intestinal tissues prior to cutting longitudinally and then processed separately.

6 Vortex each tube for 10 sec. on level 6, and then decant the contents onto a mesh filter or cell strainer inserted into a 50 ml centrifuge tube.

The filtered liquid will contain a single cell suspension of the epithelial cells released by the first treatment with EDTA and DTT. The remaining intestinal tissue will be retained by the mesh filter/cell strainer.

7 Using a sterile forceps, transfer the intestinal pieces to a fresh tube containing 4 mL of RP5/EDTA/DTT and incubate shaking (250 rpm) at 37°C for 10 min. Repeat this process one more time for a total of three (20 min., 10 min., & 10 min.) EDTA/DTT treatments. Store the pooled filtrates on ice.

8 Transfer the intestinal pieces to a fresh tube containing 5 ml of RP5/HEPES. Incubate shaking (250 rpm) at 37°C for 5 min.

This step serves to rinse the tissue to remove excess EDTA/DTT, however, the remainder of the epithelial cells will also be released during this wash.

9 Decant the tissue pieces over a mesh filter/cell strainer. If isolation of lamia propria cells is desired, save the tissue pieces and set aside. Pool the RP5/EDTA/DTT and RP5/HEPES washes (total volume should be ~17 mL) and centrifuge at low speed (1,200 × *g*) to pellet the cells.

10 To quantify extracellular bacteria, collect the supernatant and centrifuge for 20 min at 12,000 × *g*. Suspend the bacterial pellet in 0.5 mL of sterile water and store on ice.

Alternatively, the total number of bacteria (extracellular plus intracellular) in the EC fraction can be determined by omitting the low speed spin.

11 To quantify intracellular bacteria, suspend the epithelial cells in 5 mL of RP5/$Gent_{25}$. Incubate the single cell suspension for 20–30 min at 37°C in a CO_2 incubator.

The gentamicin will not penetrate the mammalian cell, and therefore will only kill any residual extracellular bacteria or bacteria attached to the EC.

12 Wash the cells twice in sterile PBS. Re-suspend the cells in 0.5 mL sterile water, vortex for 30 s to lyse the cells, and then store on ice.

Isolation of lamina propria (LP) cells

13 Transfer the intestinal pieces to a 50 mL tube containing 4 mL of digestion solution. Add a sterile stir bar and incubate shaking (250 rpm) at 37°C for 30 min.

14 Pulse the tissues by vortexing each tube as follows: 5 s on level 5; then 10 s on level 7; then 5 s on level 8; 5 s on level 5 and 5 s on level 7.

15 Decant the solution onto a mesh filter or cell strainer inserted into a 50 ml centrifuge tube. Transfer the undigested pieces and the stir bar into a fresh tube containing 4 mL digestion solution and store the filtrate on ice.

The filtrate contains released LP cells.

16 Incubate the tissue pieces for 30 min. at 37 °C shaking at 250 rpm. Decant the solution through the same mesh filter and pool the filtrate with the previous filtrate.

At the end of the second incubation, the intestinal tissues should be completely digested. If residual tissue remains, and additional 30 min. incubation in fresh digestion solution can be performed.

17 To quantify extracellular bacteria, collect the supernatant and centrifuge for 20 min at 12,000 × *g*. Suspend the bacterial pellet in 0.5 mL of sterile water and store on ice.

Alternatively, the total number of bacteria (extracellular plus intracellular) in the LP fraction can be determined by omitting the low speed spin.

18 To quantify intracellular bacteria, suspend the LP cells in 5 mL of RP5/$Gent_{25}$. Incubate the single cell suspension for 20–30 min at 37°C in a CO_2 incubator.

Gentamicin used at this concentration will not penetrate mammalian cells, and therefore will only kill any residual extracellular bacteria or bacteria attached to LP cells.

19 Wash the cells twice in sterile PBS. Re-suspend the cells in 0.5 mL sterile water, vortex for 30 s to lyse the cells, and then store on ice.

Enumeration of bacteria

20 Prepare serial 10-fold dilutions of each processed mucus, EC, or LP sample in sterile water (50 µl of sample into 450 µl of water). Spread 50 µl samples of appropriate dilutions onto one-third sections of a BHI/L+G agar plate. Incubate for 36–48 h at 37°C.

Tiny colonies will be visible after 24–30 hours of incubation, but it will be easier to distinguish *L. monocytogenes* colonies (creamy white, dome-shaped) from any intestinal microbiota that are not inhibited by the selective agar after 48 h. Suspect colonies can be verified by plating on *Listeria* ChromAgar.

21 Calculate the CFU/ml by multiplying the number of *L. monocytogenes* colonies observed by 20 (since only 50 µl was plated) times the dilution factor. The total CFU per tissue can be determined by multiplying the CFU/ml by the total volume of the sample.

BASIC PROTOCOL 4 ENUMERATION OF DISSEMINATED BACTERIA

During the first 36 to 48 hours after food borne transmission, *L. monocytogenes* are found only in the gut tissue (Bou Ghanem et al., 2012). At later time points during the infection, bacterial loads can also be determined in the spleen, liver, gall bladder and brain.

Materials

Infected 6- to 9-week old mice (Basic Protocol 1)

70% ethanol in a spray bottle

Sterile dissection instruments including:

Medium dissection scissors

Tissue forceps

Small surgical scissors

Styrofoam or cork dissection board and dissecting pins

polypropylene centrifuge tubes, labeled and filled with sterile water

microcentrifuge tubes, filled with 1 ml water, sterile

Tissue Homogenizer (PowerGen1000 or similar)

50 ml polypropylene centrifuge tubes, sterile, filled with ~ 25 ml sterile H_2O

50 ml polypropylene centrifuge tubes, sterile, filled with ~ 25 ml 70% ethanol

1.5 ml microcentrifuge tubes, sterile, filled with 450 μl of sterile H_2O

BHI/L+G agar plates (see Support Protocol 2)

37 °C incubator

1 Euthanize the infected mouse and thoroughly spray the abdomen with 70% ethanol to disinfect and wet the fur.

If using multiple groups of animals, it is most efficient to euthanize all of the mice (up to 4 animals) in the experimental group at once.

2 Pin the limbs of each mouse to a dissecting board. Grab the skin with a pair of forceps, and then use medium dissection scissors to make a midline incision in the skin along the length of the mouse. Peel back the skin to expose the peritoneum and pin down each skin flap.

3 Using small surgical scissors, cut through the peritoneal wall to expose the internal organs.

Gall bladder, spleen, and liver harvest

4 Locate the gall bladder (a small yellow fluid-filled sac attached to the liver) and snip carefully with sterile scissors to detach from liver without bursting. Transfer to a microcentrifuge tube containing 1 mL sterile water.

5 Aseptically remove the liver, making sure to remove all lobes, and transfer to a centrifuge tube containing 2.5 mL of sterile water.

6 Grasp the spleen with sterile forceps and liberate by make two cuts, one at each end. Place in a centrifuge tube containing 2.5 mL sterile water.

Brain harvest

7 To harvest the brain, flip the mouse over and cut the skin and tissue above the neck and across both ears at a 45° angle.

8 Peel off a U-shaped flap of skin by pulling towards the nose with sterile forceps, exposing the skull and facial bones.

9 Hold the bony ridge between the eyes with a pair of forceps, and then make shallow cuts across the lateral edges of the skull with a pair of surgical scissors.

Be careful to cut only the bone and not the brain tissue.

10 Cut the bony ridge between the eyes. Use a forceps to hold the top of the skull and pull backwards (toward the neck) to expose the brain. Gently lift the brain from its cavity using sterile forceps and place in a centrifuge tube containing 1.5 mL of sterile water.

Tissue homogenization

11 To process the gall bladders, use sterile scissors to tear apart the tissue in the microcentrifuge tube, and then vortex vigorously for 30 sec. Prepare serial dilutions and plate on either BHI/L+G agar.

12 For all other tissues, prepare homogenates using a tissue homogenizer.

Disinfect the homogenizer by placing the probe in a 50 ml centrifuge tube containing ~25 ml of 70% ethanol. Homogenize at 60% power for 10–15 sec.

Wait for the ethanol to completely air dry, or place the probe in another 50 ml tube containing ~25 ml of sterile H_2O and process at 60% power for 10–15 sec. to dilute the ethanol.

13 Homogenize each tissue for 30 sec. at 60% power. Clean the probe between tissue samples by homogenizing for 10–15 sec. in sterile water and disinfect with 70% ethanol between sample groups.

Use a separate water tube for each sample group and tissue type (e.g. colons from experimental group A animals).

14 Prepare serial 10-fold dilutions of each sample in sterile water (50 µl of sample into 450 µl of water). Spread 50 µl samples of appropriate dilutions onto one-third sections of either BHI or BHI/L+G agar plates. Incubate for 36–48 h at 37°C.

Tiny colonies will be visible on BHI/L+G after 24–30 hours of incubation, but it will be easier to distinguish *L. monocytogenes* colonies (creamy white, dome-shaped) from any intestinal microbiota that are not inhibited by the selective agar after 48 h. Some inhibition of listerial growth may be noted for undiluted liver samples plated on BHI/L+G agar. Suspect colonies can be verified by plating on *Listeria* ChromAgar.

15 Calculate the CFU/ml by multiplying the number of *L. monocytogenes* colonies observed by 20 (since only 50 µl was plated) times the dilution factor. The total CFU per tissue can be determined by multiplying the CFU/ml by the total volume of the sample.

SUPPORT PROTOCOL 1 PREPARATION OF CONTAMINATED BREAD PIECES

A key feature of the food borne listeriosis model is that each mouse picks up a piece of contaminated food and eats the entire piece. The following protocol describes the process of

contaminating small pieces of commercially available sliced bread with *L. monocytogenes* in a manner that reproducibly yields a consistent inoculum for each animal.

Materials

White bread, cut into small (2–3 mm) cubes & stored individually in sterile microcentrifuge tubes

Salted butter, stored as small (0.5–1 cm) chunks in sterile microcentrifuge tubes

An aliquot of late log phase growth of *L. monocytogenes*, titered & stored at −80°C (see recipe)

Brain Heart Infusion (BHI) broth

Sterile 125 ml flask

Sterile Phosphate buffered saline (PBS)

1. Prepare bacteria by thawing an aliquot of intestinally passaged *L. monocytogenes* on ice. Add the desired amount to 10 ml of BHI broth in a 125 ml flask, and incubate at 30°C for 1.5 h.
2. About 20 min. before the bacterial culture is ready, thaw bread pieces at room temperature, melt an aliquot of butter at 55 °C, and pre-warm a small volume of PBS at 55°C.

   Prepare enough materials to have at least one bread piece per mouse and at least one extra piece for determining the titer of the actual inoculum.
3. Based on the predetermined titer of the bacterial aliquot, calculate the total volume of *L. monocytogenes* broth culture needed to contaminate all of the required bread pieces. Pellet the bacteria by centrifuging at 14,000 × g for 10 min. Aspirate all BHI broth and suspend the bacteria in a small volume of pre-warmed PBS (2 μl per bread piece).
4. Vortex the melted butter, and add the required volume (3 μl per bread piece) to the bacteria suspended in warm PBS. Mix thoroughly.

   It is best to work in small batches (enough for no more than 8–10 bread pieces at a time) so the butter does not solidify before it can be transferred to the bread pieces. Use of butter results in a more consistent rate of infection in mice.
5. Working quickly, pipet 5 μl of the bacteria/PBS/butter suspension onto a single bread piece within a microcentrifuge tube.

   The solution should be completely absorbed by the bread piece.
6. To determine the actual titer of the prepared inoculum, add 1 ml of sterile PBS to one of the contaminated bread pieces. Vortex the tube vigorously for 1 min. Prepare serial dilutions and plate on both BHI and BHI/L+G agar.

SUPPORT PROTOCOL 2 PREPARATION OF SELECTIVE GROWTH AGAR MEDIA (BHI/L+G)

Growth of the gut microbiota must be inhibited in order to quantify the number of *L. monocytogenes* CFU present in intestinal tissue homogenates. The selective agar used in this protocol is referred to as BHI/L+G and is a variation of McBride's agar (McBride and Girard, 1960). The selective properties of the lithium chloride and glycine contained in the medium prevent the growth of most intestinal microbiota, at a density of up to 10^7 CFU/ml. It is important to note that his media does not support the growth of all *L. monocytogenes* strains. For example, *L. monocytogenes* EGDe grows well on this agar, with colonies visible within 36–48 hours, but *L. monocytogenes* 10403s does not grow on this media. For isolation of *L. monocytogenes* strain 10403s, BHI containing 10 μg/ml streptomycin is better choice.

Materials

Brain Heart Infusion (BHI) agar (Difco)

Lithium chloride (LiCl)

Glycine

1 L flask

Sterile petri dishes

De-ionized water

1. Place 26 g of BHI agar, 7.5 g of LiCl and 5.0 g of glycine in a 1 L flask and then add 500 ml of deionized water and a magnetic stir bar.
2. Heat until the agar boils, stirring continuously.

   To ensure that the agar goes into solution completely, take the flask off the heat once it starts to boil and let the boiling subside. Swirl vigorously, and then place the flask back on the heat and bring the solution to a boil again.
3. Autoclave the media for 30 min at 121°C under 16–19 psi on a liquid cycle.

   Leave the stir bar in the flask.
4. Equilibrate the media to ~55–60°C in a water bath.

   Do not allow the solution to cool to less than 55°C or crystals will form in the agar. Agar containing crystals will support the growth of *L. monocytogenes* and will inhibit growth of the intestinal microbiota. However, the crystals will be similar in appearance to tiny colonies and will make it difficult to count bacterial CFU.
5. Gently mix the agar for 1 min using a magnetic stirrer and then pour into sterile petri dishes (~ 25 ml per dish).

   Avoid prolonged or vigorous mixing so that air bubbles are not generated in the agar.

REAGENTS AND SOLUTIONS

Digestion solution

250 U/ml collagenase type IV (Worthington)

40 μg/ml DNAse I

Dissolve in RP5/HEPES

Filter sterilize

Prepare fresh

Intestinally passaged late log phase growth L. monocytogenes aliquots

Grow *L. monocytogenes* in BHI broth shaking at 30°C until the culture reaches an OD_{600} between 0.8 and 1.0. Prepare 525 μl aliquots in microcentrifuge tubes and store at −80 °C. To titer the bacterial aliquots, thaw a tube on ice, then add 500 μl to 9.5 ml BHI broth. Incubate for 1.5 h standing at 30°C. Plate serial dilutions on BHI agar. Bacterial titers should be between 1 to 20 × 10^8 CFU/ml.

Vortex the culture frequently to ensure preparation of homogeneous aliquots. The expected yield from each tube should vary no more than 2 to 4-fold.

RP5/EDTA/DTT

188 ml RPMI 1640

10 ml fetal bovine serum, heat-inactivated (5% final; Gemini)

2 ml 0.5 M EDTA (5 mM final)

Add 30.8 mg DTT (1 mM final)

Filter sterilize

Prepare fresh

RP5/HEPES

93 ml RPMI 1640

5 ml fetal bovine serum, heat-inactivated (5% final; Gemini)

2 ml of 1M HEPES (20 mM final)

Store up to 4 weeks at 4°C

RP5/$Gent_{25}$

95 ml RPMI 1640, sterile

5 ml fetal bovine serum, heat-inactivated, sterile (5% final; Gemini)

0.05 ml 50 mg/ml gentamicin (25 μg/ml final)

Store up to 4 weeks at 4°C

6 mM N-acetylcysteine (NAC)

979 mg NAC (Sigma #A-9165)

Add PBS to 100 ml

Filter sterilize

Prepare fresh

COMMENTARY

Background Information

Listeria monocytogenes are facultative intracellular bacteria that cause food borne infections in humans with a wide range of clinical outcomes, from mild, self-limiting gastroenteritis to life-threatening meningoencephalitis (Allerberger and Wagner, 2010). The most commonly used animal model of listeriosis is intravenous infection of mice (Cabanes et al. 2008). Although i.v. inoculation results in highly reproducible infections of the spleen and liver, this route of administration completely bypasses the gut mucosa.

Oral transmission of *L. monocytogenes* has been used less frequently because it was widely believed that mice were resistant to infection by this route. Mouse resistance was largely attributed to a low affinity interaction between the bacterial surface protein internalin A (InlA) and murine E-cadherin expressed on intestinal epithelial cells (Lecuit et al., 1999). To overcome this limitation, investigators have either modified the host by generating transgenic mice that express human E-cadherin (Disson, O. et al, 2008), or engineered the bacteria to express a modified InlA protein that has a higher affinity for murine E-cadherin (Wollert et al., 2007).

Even with these advances, however, the primary route of transmission used by investigators continued to be intragastric (i.g.) inoculation, an invasive procedure that deposits *L. monocytogenes* suspended in saline directly into the stomachs of fasted mice. Intragastric inoculation has three primary disavantages. First, the inoculated bacteria transit very rapidly through the intestinal tract, with the majority of the inoculum excreted in feces within three hours (Hardy et al., 2004). This means that the bacteria will have only limited exposure to the low pH and high osmolarity of the upper GI tract, and thus, may not be as “gut adapted” as bacteria that reside on contaminated pieces of food and undergo prolonged digestive processes in the stomach. Second, the invasive nature of the procedure may cause tissue damage that leads to rapid invasion of the blood stream without crossing the gut mucosa. This introduces variability that is investigator-dependent, as evidenced by the fact that many labs have reported that i.g. inoculation results in systemic spread within as little as 4 hours (Czuprynski et al., 2003; Gajendran et al., 2007; Lecuit et al., 2001; Wollert et al., 2007) while other groups found no systemic spread for up to 48 hours after inoculation (Kursar et al., 2004; MacDonald and Carter, 1980; Monk et al., 2010). Finally, a common feature of i.g. infection with many oral bacterial pathogens, including *L. monocytogenes*, has been an general acceptance of a wide degree of spread in CFU counts obtained for intestinal tissues. The variable bacterial loads may be related to the degree of early systemic spread in each

animal or may be a result of re-inoculation when mice ingest contaminated feces shed into their cages throughout the course of the infection.

The natural feeding model described here offers several advantages over the i.g. infection model. Food borne transmission is not physically invasive, and thus, poses no risk for unintended trauma to the lining of the esophagus or stomach. The method does not involve specialized skills on the part of the investigator, and therefore, should reduce lab-to-lab variation in the kinetics of systemic spread. The food borne listeriosis model is ideal for studying host immune response to infection since any mouse strain, including specific gene knockout and transgenic animals can be used, a key advantage compared to the recently described guinea pig model (Melton-Witt et al, 2011). Finally, the techniques described here should be readily adaptable to other orally transmitted bacterial pathogens such as *Yersinia*, *Salmonella*, or *Citrobacter* species.

Critical Parameters

It is essential that all *L. monocytogenes* strains to be used as bacterial inocula are cultivated using the same culture conditions including the size of the flask used, the type of media, the incubation temperature, and the amount of aeration.

This model is also ideally suited to test the infectivity of *L. monocytogenes* propagated on various food sources. Bread pieces were chosen for this protocol because they readily absorb a liquid inoculum. The bacterial inoculum can also be suspended in plain PBS; however, we found that the use of melted butter resulted in increased survival of the inoculum and more consistent levels of infection in the gut (Bou Ghanem et al., 2012). Likewise, fasting the animals prior to infection also resulted in significantly increased levels of *L. monocytogenes* colonization in the intestines.

When harvesting both gut tissues and peripheral organs (Basic Protocols 3 & 4), collecting the organs in a particular order can be helpful. It is easy to accidently rupture the gall bladder, so it should be removed first. The MLN are most readily identified when the intestines are in their natural locations, so they should be collected second. Delineation of intestinal tissues such as the ileum or colon is best achieved by harvesting the entire length of the intestinal tract, and then using the stomach and cecum as reference points for further dissection. The spleen and liver are removed next, and the brain is harvested last, as it will be necessary to flip the carcass over to access the skull.

To obtain CFU counts that accurately reflect the bacterial loads in vivo, it is critical to work quickly, limiting the amount of time spent processing each sample. After harvesting from infected animals, all tissues should be stored on ice throughout the processing steps to reduce bacterial growth in vitro. Ideally, all tissue samples should be homogenized, then serial dilutions for all samples should be prepared, with plating of all dilutions occurring as the last step. This will allow for direct comparison of CFU counts as the amount of time spent processing the tissues will be the same for all groups.

Troubleshooting

Inbred strains of laboratory mice are not equally amenable to feeding at any time of the day (Kowal et al., 2002). The likelihood that a mouse will readily eat the contaminated bread piece varies with both the strain type and the age of the mice. In our experience, 6–9 week old C57BL/6 mice are receptive to feeding at most times during the normal work day. However, up to half of the BALB/c/By/J mice offered a contaminated bread piece during the day will not eat it. This issue can be resolved by infecting the mice during the evening (9–10 PM), when all animals will readily accept the bread piece. Alternatively, the light cycle of the room used to house the animals can be changed so that the dark phase coincides with the normal working hours for laboratory personnel. Some animal facilities will also have cabinets available that can be maintained on a different light cycle than the room they are housed in. If animals need to be moved to housing with a reversed light cycle, they should be given at least two weeks to acclimate to the new schedule prior to infection. For infections that take place during the dark cycle, only red lamps should be used in either the room itself or in the laminar flow hood.

Anticipated Results

Susceptibility to food borne listeriosis varies depending on the bacterial strain used, the dose of the inoculum, and the mouse strain used. For example, female BALB/c/By/J mice are highly susceptible to *L. monocytogenes* infection while both male and female C57BL/6 mice are more resistant (Bou Ghanem et al., 2012). Infection can be established in susceptible BALB/c mice with as few as 10^7 CFU of *L. monocytogenes*. C57BL/6 mice require 10–100 fold more bacteria to establish similar colonization levels 24 hours post-infection. In either mouse strain, the bacteria begin to disseminate from the gut to the spleen and liver 36–48 hours post infection. Colonization of the gall bladder begins 3–4 days post-infection. In susceptible mice infected with a dose of 10^8–10^9 CFU, *L. monocytogenes* can be detected in the brain beginning 5 days after ingestion of the contaminated food.

If age (within 2–3 weeks) and gender-matched mice are used, and care is taken in preparing uniformly infected bread pieces, this model system can yield highly reproducible data with a variation in CFU counts of no more than 10–100-fold within experimental groups. Typically, groups of 4 mice can be infected in a single experiment, the experiment can be repeated once or twice, and the data from all 8–12 mice can be pooled together for analysis.

Time Considerations

Infection of mice requires careful advance planning. The mice should be gender and aged matched, and ideally, used between the ages of 6 to 9 weeks old. If purchased from an outside vendor, the mice should have at least 1 week to acclimate before handling. Weanlings or purchased animals that will be moved to rooms or cabinets with an altered light cycle should be given at least 2 weeks to acclimate to the new schedule before infection.

Preparation of *L. monocytogenes*-infected bread pieces takes approximately 2 hours. Typically, each mouse will pick up the bread and consume the entire piece within 2–5 minutes. Since the mice are infected individually in empty cages, the duration of this phase

of the experiment will depend on the amount of available laminar flow hood space in the investigator's animal facility. Occasionally, some mice need longer to eat the bread piece, so the investigator should be prepared to check on those animals 20–30 min. later (or up to 2 hours later).

The time required for organ recovery will depend on both the expertise of the investigator and the number of tissues being analyzed. To process the harvested tissues in a timely manner, it is best to divide the tasks involved among several individuals. It takes approximately 8–10 min. to properly flush one intestinal tissue (e.g. ileum or colon). Homogenization requires ~ 2 min. per tissue sample. Dilution and plating takes 5 min. per tissue sample.

An experienced team of two investigators can process the ileum, colon, MLN, gall bladder, spleen, and liver from 8 infected mice in approximately 4.5 hours. The rate liming steps are flushing of the intestines (because of the time involved), and homogenization (because most labs have only one homogenizer). When analyzing multiple tissues from a large number of mice, the dilution and plating steps can be expedited by involving multiple investigators. This will also ensure that all tissues are plated at approximately the same time post-harvest.

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