Video Article

Listeria monocytogenes Infection of the Brain

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Abstract

Listeria monocytogenes is an intracellular bacterial pathogen that is frequently associated with food-borne infection. The ability of *L. monocytogenes* to cross the blood-brain barrier (BBB) is concerning as it can lead to life-threatening meningitis and encephalitis. The BBB protects the brain microenvironment from various toxic metabolites and microbial pathogens found in the blood following infection, and therefore supports brain homeostasis. The mechanisms by which *L. monocytogenes* present in the bloodstream cross the BBB to cause brain infections are not fully understood and there is also a lack of a robust model system to study brain infections by *L. monocytogenes*. Here, we present a simple mouse infection model to determine whether bacteria have crossed the BBB and to quantitate the burden of bacteria that have colonized the brain *in vivo*. In this method, animals were infected intravenously with *L. monocytogenes* and were humanely euthanized by exposure to CO_2 followed by cervical dislocation. Cardiac perfusion of the animals was performed prior to harvesting infected organs. Blood was collected before perfusion and the number of bacteria per organ or mL of blood was determined by plating dilutions of the blood or organ homogenates on agar plates and counting the number of colonies formed. This method can be used to study novel receptor-ligand interactions that enhance infection of the brain by *L. monocytogenes* and can be easily adapted for the study of multiple bacterial pathogens.

Video Link

The video component of this article can be found at https://www.jove.com/video/58723/

Introduction

The Gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen and one of the most deadly food-borne pathogens worldwide. Ingestion of *L. monocytogenes* contaminated food can lead to listeriosis in humans, a severe invasive disease targeting mostly pregnant women, newborns, the elderly, and immunocompromised individuals[1]. *L. monocytogenes* is among the leading causes of death by a food-borne pathogen in the U.S. and case fatality rates from listeriosis are as high as 20–30%, the highest for all food-borne pathogens[2]. No vaccine currently exists for *L. monocytogenes* and the ability of bacteria to effectively spread to distal organs and the brain by crossing the blood-brain barrier (BBB) may lead to life-threatening meningitis and colonization of the brain[3,4,5,6]. Bacterial meningitis is typically severe; while most people who receive treatment recover, infections can cause serious complications, *e.g.*, brain damage, hearing loss, or learning disabilities in children. *L. monocytogenes* is predicted to account for at least 10% of all community acquired meningitis in the U.S.[7].

A major route for bacterial dissemination to the brain and meninges is through the bloodstream. Bacteria circulating in blood vessels in the brain are able to cross the BBB to cause brain infection. The BBB is a highly vascularized barrier system that protects the brain from foreign particles circulating in the blood. Endothelial cells constitute a layer that lines the interior surface of the blood vessels[8,9]. In addition to *L. monocytogenes*, multiple bacterial pathogens such as *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Escherichia coli*, and *Haemophilus influenzae* type b (Hib) are capable of colonizing the brain by crossing the BBB[3,4,5,6]. However, when examining bacterial burdens in the brain of infected mice, it is important to determine whether bacteria have crossed the BBB, otherwise the bacterial burden in the brain may represent bacteria that are still in the blood vessels of the brain. Thus, it is necessary to perfuse the mice of all blood prior to determining colony-forming units (CFU) of brain homogenates.

In this study, we describe *in vivo* methods to examine *L. monocytogenes* infection of the brain. For the methods described here, we used *L. monocytogenes* strain 10403S. *L. monocytogenes* 10403S is one of the most widely used laboratory strains to study systemic listeriosis in the mouse model of infection[10]. This protocol is based on standard intravenous injection of *L. monocytogenes* followed by perfusion of the mice. A schematic outline of the infection protocol in mice is shown in **Figure 1**. *L. monocytogenes*-infected brains and other organs from non-perfused or perfused mice were collected and the bacterial organ burden determined. These methods are useful for not only determining total bacterial colonization of the brain in infected animals, but are also beneficial for determining whether bacteria have penetrated the BBB *in vivo* to mediate invasion of the brain. It is important to highlight that this laboratory protocol should be conducted following consultation with the relevant institutional biosafety committee and animal facility management.

Protocol

All animals are to be maintained and handled with maximum care to minimize discomfort during the course of the procedure. The procedure is to be conducted in compliance with the Institutional Animal Care and Use Committee and all federal, state and local laws. Also note that the laboratory experiments are to be conducted in accordance with Biosafety Level 2 guidelines.

1. Growth of *L. monocytogenes* for Mouse Infection Studies

1. **Autoclave 500 mL of Brain Heart Infusion (BHI) agar medium in a 1 L Erlenmeyer flask to prepare solid media plates. Allow the media to cool in a 56 °C water bath and then add streptomycin at a final concentration of 100 µg/mL.**
    1. Pour 25 mL of media/Petri dish into Petri dish plates. Allow the plates to dry at room temperature (22 ^{o}C-25 °C). If needed, the plates can be dried at 37 °C until completely dry.

2. **Using a sterile inoculating loop and aseptic technique, obtain a loop full of frozen *L. monocytogenes* strain 10403S[11,12] from a stock culture frozen in BHI media plus 30% glycerol and maintain in a -80 °C freezer.**
    1. Streak the *L. monocytogenes* on a BHI agar/streptomycin plate such that single bacterial colonies can be isolated. Place the plates in a 37 °C incubator overnight (18 h to 24 h) to grow the *L. monocytogenes* colonies.
    2. Using a sterile inoculating loop, pick a single *L. monocytogenes* colony from the BHI agar plate and inoculate the colony into a sterile test tube containing 5 mL of BHI broth containing 100 µg/mL streptomycin.

3. **Incubate the *L. monocytogenes* culture tube slightly tilted at 30° overnight in a static incubator.**
    1. The following day, visually inspect the *L. monocytogenes* culture for growth (the BHI media will be turbid) and vortex briefly to ensure a uniform suspension of the culture.
    2. Aseptically remove a 1 mL aliquot of the bacterial culture and measure the optical density at 600 nm (OD_{600}) using a spectrophotometer.
    NOTE: Sterile BHI broth is used as the blank for the spectrophotometer reading before measuring the optical density of the *L. monocytogenes* culture. The *L. monocytogenes* culture can also be diluted (two to five-fold) in BHI before reading the optical density.
    3. Determine the number of *L. monocytogenes* colony forming units (CFU) per mL of BHI culture by plating 10-fold serial dilutions of the culture. This is useful to establish a relationship between the optical density of the *L. monocytogenes* culture and the number of bacteria per mL of culture. In general, an OD_{600} of 1.0 for a *L. monocytogenes* culture equals approximately 1 x 10^9 CFU of bacteria per mL.

2. Preparation of *L. monocytogenes* for Infection of Mice

1. Eighteen to twenty-four hours prior to animal infection, grow *L. monocytogenes* cultures in BHI broth containing 100 µg/mL streptomycin and determine the OD_{600}/~CFU per mL as indicated in step 1.
NOTE: Mice are generally ordered one week in advance of infection and are acclimated to the animal facility before infection studies are performed. In this study, 6–8 weeks old female BALB/c mice (5 mice per group) were housed in a barrier environment in the Harvard Medical School BSL2-level animal containment facility and supplied food and water *ad libitum*.
2. Determine the amount of *L. monocytogenes* culture required to infect each mouse based on the ~CFU per mL of bacterial culture. Make a dilution of the *L. monocytogenes* culture in sterile phosphate-buffered saline pH 7.2 (PBS) to the appropriate concentration of bacteria. Mice are typically infected intravenously with 200 µL of PBS containing 1–2 × 10^4 CFU of bacteria/animal.
3. Verify the number of *L. monocytogenes* in the inoculum by plating 10-fold serial dilutions onto BHI agar plates containing 100 µg/mL streptomycin. Incubate the plates in a 37 °C incubator overnight to determine the number of *L. monocytogenes* inoculated per mouse as follows:
Inoculum (CFU) = (number of colonies x dilution factor) / mL plated x volume (mL) injected

3. Infection of Mice with *L. monocytogenes via* Intravenous Tail Vein Injection

1. **Remove the lid and food bins from the cage containing the mice, and place the cage under a 250 W infrared heat lamp for 5 min.**
NOTE: This method allows the tail veins to dilate, ensuring that injections are easier to perform.
    1. Carefully place the animal in an appropriately sized mouse restraining device to safely restrain the animal during tail vein injections.

2. Mix the *L. monocytogenes* inoculum (step 2.2) and load the inoculum into a 1 mL syringe equipped with a 26 G needle.
3. Locate a lateral tail vein of the mouse and clean the injection site using an alcohol pad or a 75% ethanol spray.
4. **Gently inject the mouse with 200 µL of the *L. monocytogenes* suspension into a lateral tail vein using the syringe with the 26 G needle.**
    1. Use cotton gauze to briefly apply pressure on the injection site to stop any bleeding, and place the animal in a new cage.
    NOTE: Perform this process for all of the mice to be injected and mark the cage with appropriate labels. To determine the post-injection concentration of the *L. monocytogenes* inoculum, repeat step 2.3 using the remaining amount of inoculum. The intravenous *L. monocytogenes* infection inoculum per mouse is reported as the average CFU measured from the pre- and post-injection inoculum samples.

5. Monitor the infected animals daily and note any overt signs of illness (*e.g.,* ruffled fur, hunched posture, sluggish movement, weight loss). Remove animals that appear to be significantly moribund prior to 72 h post-infection (*e.g.,* 24, 48 h post-infection) from the study and humanely sacrifice. Continue daily monitoring until all remaining animals are sacrificed at 72 h post-infection.

NOTE: The 50% lethal dose (LD_{50}) for *L. monocytogenes* strain 10403S in BALB/c mice is ~1–2 x 10^4 CFU/animal. Mice infected with LD_{50} doses of *L. monocytogenes* using this protocol may exhibit signs of illness, as indicated above, and investigators could expect the mice to start succumbing to the infection following 72 h post-infection.

4. Dissection and Cardiac Perfusion of Mice Infected with *L. monocytogenes*

1. **At 72 h post-infection (or at an earlier desired time point), euthanize the infected mice using a protocol approved by the institutional Animal Ethics Committee, such as CO_2 asphyxiation followed by cervical dislocation.**
   NOTE: If blood collection is to be performed, minimize tearing of the blood vessels while performing cervical dislocation.
   1. Confirm mice have been euthanized by the absence of a paw-twitch response.
      NOTE: If cardiac perfusion is to be performed, set up an automated pump/perfusion system at a flow rate of 4 mL volume/min.
2. Place the animal on its back in a dissection pan and apply 75% ethanol on the abdominal fur/skin surface.
3. Using scissors, make an incision in the abdominal wall and expand the incision to just below the rib cage.
4. Expose the diaphragm and visceral organs.
   NOTE: Be careful not to lacerate any visceral organs.
5. Open the thoracic cavity by cutting the diaphragm and cut the rib cage bilaterally to expose the left ventricle of the heart.
6. Using blunt forceps, gently grasp the heart and insert a 21 G butterfly needle connected to a perfusion system into the left ventricle and then carefully cut the right atrium to collect the blood (~0.2 mL) into a 2 mL tube containing 10 mM ethylenediaminetetraacetic acid (EDTA) to prevent coagulation. A schematic diagram of cardiac perfusion in the mouse is shown in **Figure 2**.
   NOTE: If perfusion is not to be performed, blood may be collected by cardiac puncture using a 1 mL syringe and quickly transferred into a 2 mL tube containing 10 mM EDTA to prevent coagulation. *L. monocytogenes*-infected organs are then harvested as described (step 5.1).
7. Begin perfusion and perfuse the animal for 4 min with 15–20 mL of PBS containing 10 mM EDTA.
   NOTE: Assess perfusion by observing the blood and PBS-EDTA solution flowing through the right atrium and into the dissection pan. The brain and liver will appear blanched after complete perfusion ensuring that remaining bacteria are from the harvested organs and not the circulating blood.

5. Organ Harvesting and Determination of Bacterial Burdens

1. Following perfusion, harvest organs (*e.g.,* liver, spleen) by carefully removing any vasculature and ligaments attached and place the organs separately into a sterile 15 mL conical tube containing 5 mL of sterile cold (4 °C) PBS. Store samples on ice until further processing occurs.
2. **To harvest the brain, decapitate the head behind the ears using scissors and cut the scalp skin in-between the animal's eyes down the midline. If required, trim excess tissue keeping the scissors pressed against the skull.**
   1. Gently insert one tip of the scissors into the foramen magnum (the hole in the base of the skull through which the spinal cord passes) and cut laterally into the skull on both sides.
   2. Gently cut the skull in-between the rodent's eyes down the midline, being sure to apply lateral pressure. Avoid perturbation of the brain and maintain minimum contact between the brain tissue and the scissors.
   3. Using fine tip forceps, open the skull to expose the brain and place a spatula between the underside of the brain and the base of the skull to remove the brain.
      NOTE: This results in tearing of brain nerve fibers.
   4. Carefully transfer the brain to a 15 mL conical tube containing 5 mL of sterile cold PBS.
   5. Discard the mouse carcass, and repeat these steps for the remaining animals.
      NOTE: Once all organs have been harvested, clean the procedure area and prepare for the organ homogenization to determine bacterial burdens.
3. Clean and sterilize the tip of a tissue homogenizer placed in a biosafety cabinet by inserting the tip and running the homogenizer for 10 s sequentially in 5% bleach, sterile water, 75% ethanol, sterile water each contained in a separate 15 mL conical tube.
4. **Homogenize the infected brain (~20 s on setting 6, maximum rpm) in the 15 mL conical tube until no visible tissue fragments remain.**
   1. Clean the homogenizer as described in step 5.3 to prevent bacterial carry over contamination to the next organ sample.
   2. Perform the homogenization process for all other organs (*e.g.,* liver, spleen).
   3. Once the homogenization process is completed, clean the homogenizer as described in step 5.3 and store until further use.
5. **Prepare 10-fold serial dilutions of the organ homogenates in sterile PBS and plate the dilutions on BHI agar/streptomycin plates to determine the CFU per organ.**
   NOTE: A similar method is performed to determine the CFU per mL of blood.
   1. After all dilutions are plated, transfer the plates to a 37 °C incubator overnight.
   2. The following day, count the number of colonies on each plate to determine the total number of bacteria per organ or mL of blood as follows:
      CFU/organ = (number of colonies x dilution factor) / mL of homogenate plated x 5
      CFU/mL of blood = (number of colonies x dilution factor) / mL of blood plated

Representative Results

The brain is highly vascularized and *L. monocytogenes* is known to infect cell types present in the blood[3,13]. The described protocol is used to demonstrate the ability of *L. monocytogenes* to cross the blood-brain barrier (BBB) leading to infection of the brain in mice. To determine if bacteria have crossed the BBB *in vivo*, perfusion of blood in the mouse is performed prior to determining bacterial burdens in the brain. Otherwise, the CFU obtained may include bacteria that are present in the blood vessels of the brain. *L. monocytogenes* infected brains (**Figure 3A**) and livers (**Figure 3B**) before or after perfusion at 72 h post-infection is shown. **Figure 4** shows the bacterial burdens in *L. monocytogenes*-infected mouse organs and illustrates the number of bacteria present in the brain, blood, liver, and spleen of each mouse. These data suggest that perfusion of animals did not significantly affect the bacterial burden in the mouse organs examined in this study (**Figure 4**).

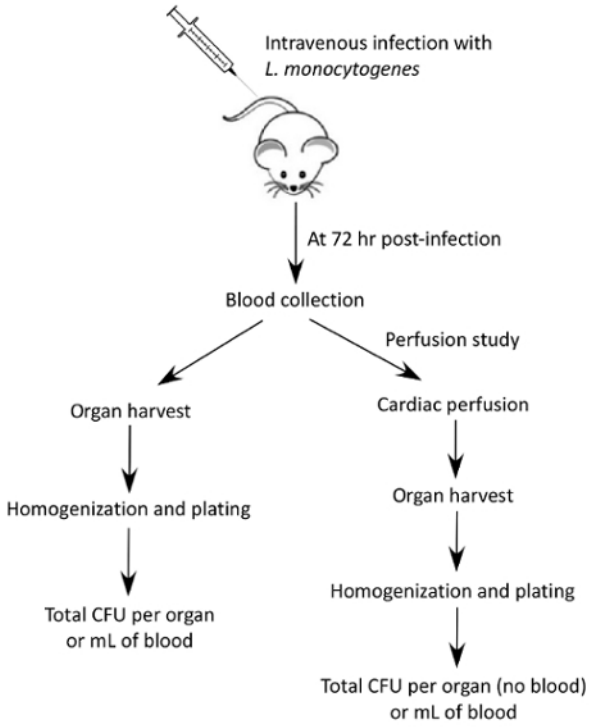

Figure 1: Schematic outline of the *L. monocytogenes in vivo* infection protocol. Please click here to view a larger version of this figure.

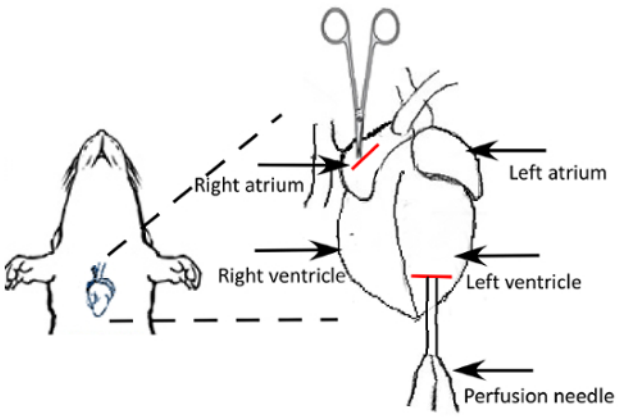

Figure 2: Schematic diagram of the cardiac perfusion procedure. Perfusion through the mouse heart showing insertion of the perfusion needle in the left ventricle (step 4.6). Following needle insertion, an immediate incision is made into the right atrium to start the perfusion procedure. Please click here to view a larger version of this figure.

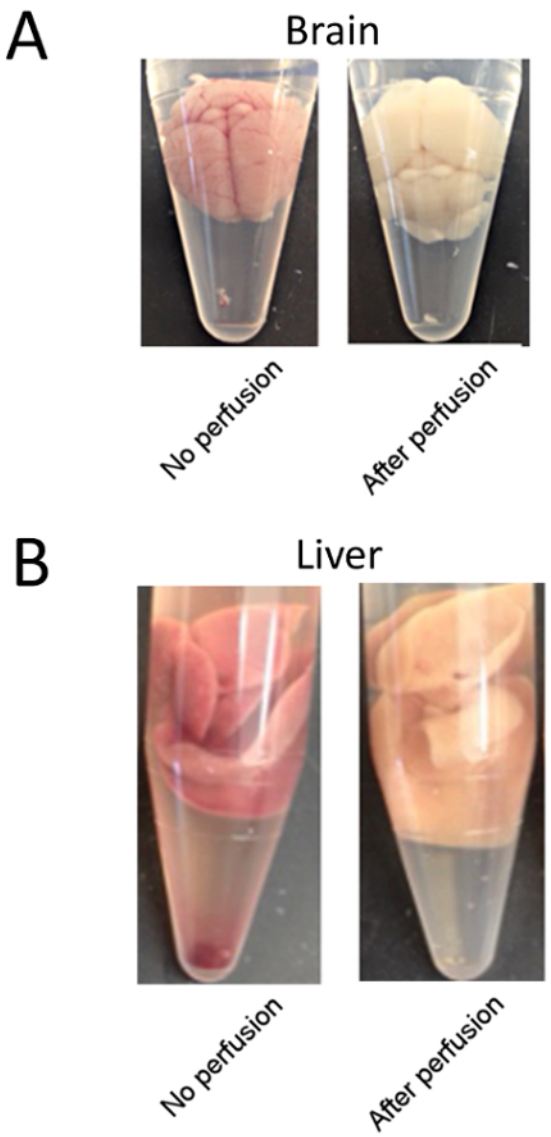

Figure 3: Harvested mouse organs following infection with *L. monocytogenes*. BALB/c mice were infected intravenously *via* lateral tail vein injection with wild-type *L. monocytogenes* 10403S, (1-2 x 10^4 bacteria/animal). At 72 hours post-infection, mice were euthanized and mouse organs were collected or euthanized mice were perfused through the heart with 15-20 mL of PBS containing 10 mM EDTA prior to organ harvesting. Representative brains (**A**) and livers (**B**) are shown from non-perfused or perfused mice. Note that the mouse organs will appear white/pale (blanched) after perfusion assuring that bacterial CFU are from the harvested organ tissue and not the circulating blood within the tissue. This figure has been modified from Ghosh *et al.*, 2018[14]. Please click here to view a larger version of this figure.

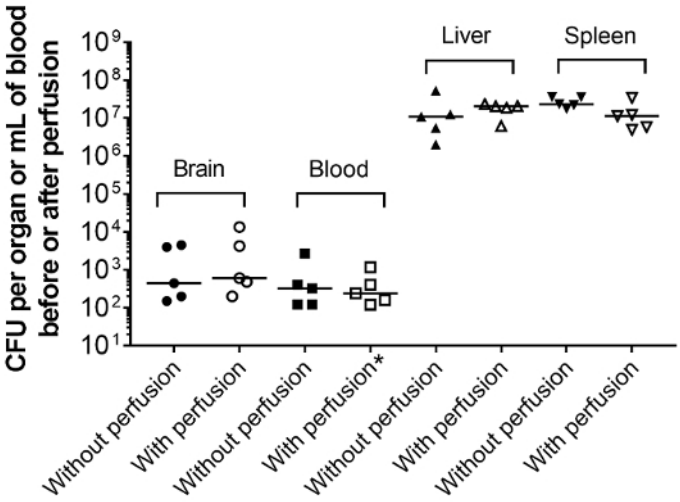

Figure 4: Bacterial burdens in infected mouse organs. BALB/c mice were infected intravenously with wild-type *L. monocytogenes* 10403S as described in **Figure 3**. At 72 h post-infection, the brain, blood, liver, and spleen of each mouse were collected and the bacterial burdens determined. In separate experiments, whole body perfusion of mice was performed and the bacterial burden within each organ determined. Horizontal lines indicate median values. * For this group, blood was collected immediately before the start of cardiac perfusion. This figure has been modified from Ghosh *et al.*, 2018[14]. Please click here to view a larger version of this figure.

Discussion

L. monocytogenes is able to cause life-threatening meningoencephalitis in humans. Prior studies have demonstrated the ability of bacteria to cross the blood-brain-barrier (BBB) and to colonize the brain. Three routes of brain invasion have been proposed during infection: direct penetration of the BBB by bacteria, stealth transport by bacteria contained inside of mononuclear cells[3], and axonal migration by *L. monocytogenes* strains that cause rhombencephalitis[15]. Since the brain is highly vascularized and *L. monocytogenes* is known to circulate in the blood during systemic infection, determination of the extent *L. monocytogenes* is able to penetrate blood vessels to colonize the central nervous system and brain is critical.

In the described protocol, intravenous tail vein injection is used to establish a systemic *L. monocytogenes* infection in mice. This method is useful to bypass the intestinal barrier and to assess specifically bacterial invasion of the BBB from the bloodstream. The protocol describes several important parameters. One important parameter is the use of the appropriate bacterial infection dose during *in vivo* experiments. This is critical to be able to compare bacterial CFU obtained from different animal groups infected with *L. monocytogenes*. Another important aspect to consider is the *L. monocytogenes* strain used for experimental study. Multiple reports have suggested differences among various *L. monocytogenes* strains in their pathogenicity and ability to infect the brain[10,16].

The protocol described here can be modified to facilitate examination of other aspects of *L. monocytogenes* infection biology. The *L. monocytogenes* infected organs can be further processed for histopathological analyses to observe visible inflammatory changes in the infected mouse organs compared to uninfected control animals. The methods described can be applied to further characterize the disease phenotypes relevant to *L. monocytogenes* strains involved in human infections as well as strains harboring defined mutants in potential virulence determinants. Such an application was recently performed to reveal a novel receptor-ligand interaction that enhances infection of the brain by *L. monocytogenes* and furthered highlighted the importance of host cell surface vimentin in host-pathogen interactions[14].

Disclosures

The authors declare no competing financial interests.

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