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Adult Zebrafish model of streptococcal infection

Hilary A. Phelps,
Assay Designs, Inc., Ann Arbor, MI, Phone: 734-668-6113, Fax: 734-668-2793, hphelps@assaydesigns.com

Donna L. Runft, and
Wayne State University School of Medicine, Detroit, MI, 313-577-9758, 313-577-1155, ak1541@wayne.edu

Melody N. Neely
Wayne State University School of Medicine, Detroit, MI, 313-577-1314, 313-577-1155, mneely@med.wayne.edu

Abstract

Streptococcal pathogens cause a wide array of clinical syndromes in humans, including invasive systemic infections resulting in high mortality rates. Many of these pathogens are human specific, and therefore difficult to analyze in vivo using typical animal models, as these models rarely replicate what is observed in human infections. This unit describes the use of the zebrafish (*Danio rerio*) as an animal model for streptococcal infection to analyze multiple disease states. This model closely mimics the necrotizing fasciitis/myositis pathology observed in humans from a *Streptococcus pyogenes* infection. The use of a zoonotic pathogen, *Streptococcus iniae*, which replicates systemic infections caused by many streptococcal pathogens, including dissemination to the brain, is also described. Included protocols describe both intraperitoneal and intramuscular infections, as well as methods for histological and quantitative measurements of infection.

Keywords

Streptococcus; zebrafish; necrotizing fasciitis; *S. pyogenes*; *S. iniae*; infection; histology; systemic; animal model

INTRODUCTION

In vivo examination of virulence and host-pathogen interactions is fundamental to the study of microbial pathogenesis. The zebrafish model of infectious disease has proven to be a valuable resource for evaluation of pathogenic organisms. Naturally, the study of zoonotic fish pathogens, including *Streptococcus iniae*, in the zebrafish model of infectious disease is an appropriate choice for examining an infection between a pathogen and its natural host. However, the zebrafish model is not limited to fish pathogens, but rather this model has very successfully been adapted to study a variety of human pathogens, namely *Streptococcus pyogenes* and *Streptococcus agalactiae*. The similarity of the zebrafish immune system to that of humans and other vertebrates has allowed for the examination of infections with several human pathogens {Phelps, 2005 #3398}. Infection can be monitored by determining bacterial load in individual organs as well as by histological analysis of a single longitudinal section of the whole animal on a single slide.

Correspondence to: Melody N. Neely.

Three basic protocols are provided in this unit. The first, Basic Protocol 1, describes the necessary set up for administration of streptococcal infections into adult zebrafish via intramuscular and intraperitoneal injections. The preparation of streptococcal cultures is also discussed in support protocol 1. The second, Basic Protocol 2, describes the procedures for the analysis and handling of the infected tissues, and the final protocol, Basic Protocol 3, discusses the analysis of streptococcal mutants in the zebrafish.

CAUTION

The streptococcal species discussed in this unit (*Streptococcus pyogenes* and *Streptococcus iniae*) are Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms, infected animals and infected tissues during these experiments. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION

This experiment 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 all other pertinent resources (*APPENDIX 1B*) for more information. Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE

The protocols described below have been approved by the Wayne State University IACUC committee and follow officially approved procedures for proper care and use of laboratory animals.

STRATEGIC PLANNING

Safety Considerations

All of the streptococcal species discussed in this unit are human pathogens. These organisms are capable of causing diseases ranging from pharyngitis or mild skin infections to necrotizing fasciitis or meningitis. Researchers are advised to follow safety requirements for BSL-2 pathogens, including wearing personal protective equipment such as gloves, laboratory coats and eye protection. Sterile dissecting tools are treated with 95% ethanol in between each animal, and the equipment used for dissections is disinfected with 70% ethanol before and after dissections. All bacterial cultures, contaminated fish water, and beakers housing infected fish are treated with a 10% bleach solution initially and later autoclaved for full decontamination. The animal carcasses are treated as biohazardous waste and stored at −20°C before final disposal. Any accidental needle sticks or exposure of open wounds to bacterial cultures should be washed immediately and evaluated by a physician.

Housing of Zebrafish

A very thorough description of housing and maintenance of zebrafish communities has been reported in a previous unit (see unit 10B.2). However, our zebrafish set up differs from those described previously and therefore, we thought it useful to include a description here. The previously described zebrafish systems require a large initial investment of funds to install specific manufactured housing, which are designed to support a large colony of zebrafish and for easy maintenance. The zebrafish housing described here requires minimal initial investment and can be expanded over time as the situation dictates. All of the equipment can be found locally at any number of fish or pet supply stores or over the Internet. One caveat is

that the system described here requires more maintenance than manufactured zebrafish housing, but the extra maintenance can easily be shared by lab members and does not require a full-time aquaculture technician.

Materials

Glass aquariums (5, 10, 15 and 25 gallon as needed)

Aquarium heaters set at 26–28°C

Biological filters

Sponge filters powered by air pumps for small aquariums

Fluval-type canister filters for large 20 gallon aquariums

Mechanical, hang-on tank filters (with replacement charcoal filters)

Siphon attached to tubing

Light source – attached to a timer for a 14-hour light/10-hour dark cycle

The size of tank and type of filtration will depend on the age of the fish. The majority of our infection protocols utilize adult fish (6–9 months), however, since we breed our own zebrafish colony (see below), we have a wide range of zebrafish ages. Each tank is treated as an individual ecosystem with its own heater and filteration system. The advantage to this set-up is that poor water quality, contaminates, or an infectious agent in one tank will not result in spread to all tanks, as in a flow-through or re-circulating system. Aquariums are filled with tap water supplemented with Amquel (0.5 ml/gallon) to remove chlorine. Tanks are left open (no lids) for greater accessibility and have lighting fixtures suspended over a grouping of aquariums that are regulated by a timer for a 14 hour light/10 hour dark cycle.

Aquarium maintenance includes a 1/3 water change 3 times weekly after removal of excess food or fish waste from the bottom of the tanks using a siphon. The partial water change, as opposed to moving all fish to a new tank, helps to avoid fluctuations in water quality and helps to maintain the biological filter. If needed, the sides and bottom of the tank are cleaned using a long handled scrubber (found at fish stores) to remove excess growth of algae or bacteria. Disposable mesh charcoal filters can be removed from mechanical filters and rinsed under distilled water as needed, until a replacement filter is required. Water quality is assessed weekly to insure that ammonia, pH and salt concentrations are optimal. Smaller larval fish are placed in smaller aquariums (5–10 gallon) with sponge filtration, while older fish can be placed in larger aquariums the stand-alone Fluval-type filtration. Monday through Friday, adult fish are fed 3 times daily with TetraMin Tropical flakes along with hatched brine shrimp in the morning and evening (brine shrimp hatching protocol reported previously) (Cosma, Swaim et al. 2006). On weekends, fish are fed once daily with flakes and brine shrimp.

Zebrafish Breeding

Healthy fish of good color (~approximately 15 females and 10 males) are placed in a 10 gallon aquarium at approximately 4 months and used as breeders until ~1 year of age. When embryos are required, an egg trap is placed in the aquarium. An egg trap can be fashioned from a small container fitted with a false bottom (such as mesh netting attached with a rubber band and suspended over the bottom) or another container that fits inside the first that has holes in the bottom. An open space should be maintained (approximately ½ to 1 inch) between the bottom of mesh and bottom container. Place marbles in the upper container and place the egg trap in the bottom of the aquarium after the evening feeding. Mid-morning, remove the egg trap and lift out the top container or remove the mesh that holds the marbles.

The embryos will have fallen down through the marbles, through the mesh or false bottom and caught in the open space of the second container. Rinse embryos into a small mesh strainer (coffee strainer works well) under slowly running tap water to remove any debris. The embryos are then placed into a small glass bowl (a glass custard cup works well) containing de-chlorinated tap water and examined under a dissecting scope. Using a plastic disposable transfer pipet or a fire-smoothed glass Pasteur pipet, remove any unfertilized eggs (will appear opaque white), dead or damaged embryos, fish waste or food debris. Embryos are then disinfected with either bleach (0.5% bleach for 2 minutes) or Perosan (1.5 ml/liter for 1 minute), followed by 3 rinses in tap water and a final rinse with de-chlorinated tap water. Additional information on zebrafish breeding and maintenance can be found in The Zebrafish Book (Westerfield 1995).

Larval Fish Care

Embryos are kept in de-chlorinated tap water in small glass dishes that are placed in a 28°C water bath (15 × 22 × 6 inch plastic container filled with water and heated with an aquarium heater). Flat test tube racks are submerged in the water bath to hold the embryo dishes off the bottom of the container. Embryos should be examined twice daily to remove any dead embryos or those that have any evidence of fungus growth. Water should be changed twice daily until larvae hatch from chorions and begin to swim freely (referred to as “swim=up”), ~5 days post fertilization. Healthy larvae are then transferred to small plastic containers (6 × 8 × 3 inch food storage containers) in the same heated water bath. Once transferred to the larger containers, larvae are fed paramecium (growth and maintenance of paramecium described previously, (Cosma, Swaim et al. 2006)) and powdered larval food (Zeigler AP100 Larval food or AZOO 9 in 1 Arificial Rotifera) 3 times daily with water changes as needed (1–2 times daily). Newly hatched brine shrimp can be added to larvae at ~2.5 weeks post-fertilization, followed by examination under the dissecting scope to determine their ability to eat brine shrimp. When the larvae are ready to consume brine shrimp, it should be added to the feeding regimen along with the paramecium and larval food, as not all larvae develop at the same rate. At ~3 weeks post-fertilization, transfer larvae to an aquarium with enough water to cover a small foam filter (~3 inches deep) with an aquarium heater. Continue feeding brine shrimp and extra-small flakes (TetraMin flakes ground with a mortar and pestle). As fish grow, increase water level until aquarium is full (keeping water about 3 inches below top of aquarium). Mechanical filtration can be added when fish are ~1/2 inch long.

BASIC PROTOCOL 1

INFECTION OF ADULT ZEBRAFISH (DANIO RERIO)

The first protocol discusses the procedures for infecting adult zebrafish. Both intramuscular (IM) and intraperitoneal (IP) injections are presented here. The method of infecting adult zebrafish with streptococcal species was first established by Neely, et al, (Neely, Pfeifer et al. 2002) and this protocol is amendable to allow for infection with other bacterial species. Further analysis of survival and infected tissues will be addressed in the subsequent basic protocols.

Materials

Zebrafish (see Strategic Planning)

Streptococcal bacterial culture (see Support protocol 1 for preparation)

Sterilized deionized water supplemented with aquarium salts, 60mg/L

168 μg/ml Tris-buffered tricaine pH 7.0, for anesthetizing fish (3-aminobenzoic acid ethylester, Sigma) [No special permits required]

336 μg/ml Tris-buffered tricaine pH 7.0, for euthanizing fish

3/10-cc U-100 ultrafine insulin syringe with a 0.5-in.-long 29-gauge needle

Small transportable holding tank with perforated lid

Gauze tape

Hemostat

Petri dish

400 ml beakers

1.5 ml microcentrifuge tubes

Plastic spoon

Plastic dissecting board

28°C glass front incubator

Setup

1. All equipment and materials needed for the injections should be disinfected and prepared prior to pulling fish from the tank and before cultures have reached log phase.

2. On the plastic dissecting board place two Petri dish lids upside down on the board to form a platform on which to place the hemostat (Figure 1A).

3. Wrap the open jaws of the hemostat with gauze tape. The gauze should be moistened with sterile water before beginning injections. The anesthetized fish will be placed in the open jaws of the hemostat and the moist gauze tape prevents damage to the fish from the teeth of hemostat (Figure 1B).

4. Prepare dilutions of tricaine solution in the sterile deionized water supplemented with aquarium salts. One solution should be at 168 μg/ml Tris buffered tricaine, for anesthetizing fish and a second solution should be at 336 μg/ml Tris buffered tricaine for euthanizing fish (Figure 1A). Each beaker should be very clearly labeled designating the concentration and the purpose of each solution.

5. Use the bottom of a Petri dish to hold sterile deionized water supplemented with aquarium salts for the purpose of rinsing the anesthetized fish just prior to injection. Rinsing off the excess tricaine solution will aid in the recovering of the fish following injection.

6. Following injections, the fish will be housed in 400ml beakers containing 225ml of sterile deionized water supplemented with aquarium salts. Each of these beakers should be fitted with perforated lids to prevent the fish from jumping out of the beakers. Four to six fish can be housed in one beaker. To prevent excessive stress to the animals do not exceed six fish per beaker. Prepare all the beakers needed for the experiment prior to beginning the injections. Label beakers with strain, dose, injection method (IM or IP) and date.

7. As described in Support Protocol 1, streptococcal cultures are grown to log phase and serial dilutions are prepared in TP medium in 1.5 ml microcentrifuge tubes. Cultures and dilutions should be stored on ice for the duration of the experiment to ensure that bacterial growth is suspended.

Note: There should be separate fish nets designated for infected and noninfected fish to prevent any possible contamination of experimental fish or the main tanks housing the fish.

Intramuscular Injections

8. Following set up preparation for injections, fish can be retrieved from the main tanks and placed in a small transportable container (a typical plastic mouse container works well) with tank water. Approximately 20–30 fish are pulled from the tanks at a time and placed in a small holding tank that can be transported to another room for the injections. Fill this holding tank with water directly from the aquarium in which the fish are being removed to lessen stress to fish.

> Limiting the number of fish pulled at one time will limit the time at which fish are kept at room temperature. If there is a delay in the experiment, the container holding the fish should be placed in the 28°C incubator or returned to the main tank to prevent undue temperature stress to the fish. Injections should be performed in a different location than in the fish rearing room. Once the fish are injected, they never return to the fish rearing facility.

9. For those just beginning zebrafish infections, 1–2 fish should be placed in the anesthetizing tricaine solution at a time. With more experience, as many as 5–6 fish can be anesthetized. The fish will turn over and their respiration will slow. Limit the time in the anesthetizing solution to 3 minutes or less.

> Prolonged incubation in the tricaine solution may result in delayed recovery or even kill the fish. Therefore, some caution should be used when beginning injection to prevent unnecessary stress to the fish.

10. Fill the syringe and remove any air bubbles. Keep the remaining culture on ice. Once the injections are completed, the inoculum will be serially diluted and plated to enumerate the colony forming units (cfu) per ml in each inoculum. Use a separate syringe for each bacterial strain to inject 10μl of bacterial suspension per fish.

> Never recap syringes. Once syringe is filled, it can be placed on the dissecting board with the cap placed under the syringe to prop the needle up off the board.

11. Place an anesthetized fish on the open jaws of the gauze wrapped hemostat with the head of the fish positioned at the hinge of the hemostat (Figure 2A). The needle should be positioned at a 45° angle in relation to the back of the fish with the needle pointing towards the head. The injection should be in the largest portion of the dorsal muscle, immediately anterior and lateral to the dorsal fin (Figure 2B). The needle is inserted into the muscle just beyond the bevel of the needle and 10μl of bacterial suspension is injected. The needle should be held in place for approximately 3 seconds after depression of the plunger to prevent the bacterial suspension from immediately coming out of the injection site. Total time out of water should be approximately 10 seconds.

> The anesthetized fish is removed from the Tricaine solution using a plastic spoon and immediately dipped in fresh water without Tricaine to rinse any excess anesthetic off of the fish.
>
> The fish should not be fed on the day of injection. The authors have experienced that the fish do not recover well from injections if they have been fed the same day.

12. Immediately place the injected fish in the appropriately labeled beaker. The remaining fish for a particular group, usually six fish per group, will be injected one at a time and added to the same beaker.

13. Once all the fish for a specific group have been injected, place the beaker in a 28°C glass front incubator, where the fish will remain for duration of the experiment (Figure 3). The glass front allows for exposure to ambient light and observation of experiment. The fish are not fed for the duration of the experiment. The experiment should not exceed five days under these conditions.

Intraperitoneal Injections

14. Follow the same instructions for set-up and for steps 8–10 of the intramuscular injections.

15. Place an anesthetized fish in the open jaws of the gauze-wrapped hemostat with the abdomen facing up and the head of the fish positioned at the hinge of the hemostat. The pectoral fins should be used as a landmark on the abdomen. The needle is held parallel to the spine and is inserted into the midline of the abdomen posterior to the pectoral fins. Again, the needle is inserted just beyond the end of the bevel and 10μl of bacterial suspension is injected.

16. As described for intramuscular injections, following injection the fish is placed into the appropriate beaker and the remaining fish are injected. The beaker should be monitored until all fish have recovered, followed by placing in the incubator as soon as possible.

> While the injections should not be rushed, the researcher should work with purpose and be mindful of the length of time to complete each group to reduce the time at which the fish are at room temperature.

17. Serial dilutions of the inoculums (that have been saved on ice) should be plated to TP plates (refer to Support Protocol 1) without antibiotics for wild type streptococcal strains and with the appropriate antibiotics for mutant strains carrying antibiotic resistance markers. The concentration of each inoculum should be confirmed by plating.

Note: All the fish in a group should recover and swim around the beaker without difficulty before being placed in the incubator. Symptoms of discomfort include erratic swimming behavior and swimming near the surface of the water with an increased rate of respiration. If the spine has been nicked during an intramuscular injection the fish may swim on its side and show extremely erratic behavior. Distressed fish should be euthanized if they fail to recover within approximately 10–15 minutes following injection. For each experiment, a set of control fish should be injected with sterile media as a negative control for injection technique and overall monitoring of the experimental conditions.

BASIC PROTOCOL 2

DISSECTION FOR COLLECTION OF INFECTED TISSUES AND ENUMERATION OF BACTERIA

Streptococci have the ability to disseminate through tissues and multiple virulence factors have been described to play a role in this function. Some streptococcal strains naturally cause a systemic infection in humans and we have duplicated this phenotype with *S. agalactiae* and *S. iniae* in the zebrafish, including dissemination to the brain (Neely, Pfeifer et al. 2002; Miller and Neely 2004; Miller and Neely 2005; Lowe, Miller et al. 2007). Other streptococcal species cause mainly localized necrotic infections, such as *S. pyogenes* in the zebrafish (Neely, Pfeifer et al. 2002; Miller and Neely 2004; Phelps and Neely 2007). Dissection of infected tissues and enumeration of bacteria from those tissues provides quantitative data of bacterial dissemination. Depending on the time point after infection, growth of bacteria isolated from tissues gives a more accurate picture of infection than histology alone. This protocol describes the procedure for isolation of infected tissues from

the heart, spleen, gall bladder, muscle and brain of the zebrafish, although other organs could also be isolated using this procedure. Following homogenization of dissected tissues, serial dilutions can be prepared for plating on selective media for tissue-specific enumeration of bacteria. In addition, described in this protocol is the procedure for cytospin preparations from infected tissues. Cytospins provide a more rapid analysis of the local environment of the bacteria in a particular tissue than histology, by depositing a single layer of cells from the homogenized tissue on a slide for staining and microscopic visualization.

Materials

Infected zebrafish (Basic Protocol 1)

336 μg/ml Tris-buffered tricaine pH 7.0, for euthanizing fish

Plastic spoon

95% ETOH in 250ml beaker (~100ml)

Bunson burner

Personal protective equipment (lab coat, gloves and eye protection)

Sterile dissection instruments including:

- Dissecting board (Styrofoam or cork cutting board)
- Dissecting pins
- Small pointed tip surgical scissors
- Tissue forceps
- Scalpel

37°C CO_2 incubator or 37°C incubator and GasPak Jars

Tissue homogenizer (Kontes cordless motor)

Disposable pellet pestles, sterile

Phosphate-buffered saline (PBS), sterile

1.5 ml microfuge tubes, sterile

Colistin-Nalidixic Acid (CNA) agar plates

Preparation

1. Prepare the agar plates in advance, warm to room temperature and label with strain, time point and tissue.

2. Set up the dissecting area with the dissecting board, dissecting instruments, beaker of 95% ETOH and bunson burner.

3. Label 1.5 ml microfuge tubes with strain, time point, and organ and add 200 ul of sterile PBS to each tube.

Dissection of organs

4. At the desired time point post-injection, place infected zebrafish into Tricaine solution for 25 minutes to euthanize.

   Alternatively, fish may be placed in Tricaine for 5 minutes, followed by quickly removing the head with a sharp scalpel. This will depend on what organs are to be removed. Fish must be freshly killed for dissection of organs,

as tissues decompose rapidly after death and may give a false positive for bacterial dissemination. Therefore, limit the number of fish euthanized at one time if only one person is performing dissections.

5. Dissect fish immediately after euthanization. Pin the fish on the board, ventral side up. Place one pin through the bottom of the jaw and one through the tail region behind anal fins.

6. Using the surgical scissors or scalpel, make a small incision (~ 1cm) across the midline of the fish directly behind the anal fins. Place the tip of the scissors in this incision and cut parallel to the midline up to and across the gill cartilage.

Be careful to only cut the skin and the gill cartilage and not the internal organs. This step is easier if you lift slightly with the scissors as you cut to pull the skin up off the peritoneal cavity.

7. Moving back to the original incision behind the anal fins, use the scissors to make a larger cut perpendicular to the midline on either side of the midline incision, again being careful not to clip any internal organs. At this point the incision should look like an upside down “T”. Do a similar cut on either side of the gills, perpendicular to the midline. The final incision should look like a capital “I”.

8. Use the dissecting pins to pin back the skin flaps on either side to open up the body cavity.

After using each instrument, place it in the beaker of ETOH and flame before using again to maintain aseptic technique.

9. Using the forceps, carefully remove the heart, located under the gill cavity, and place in the appropriately labeled tube in PBS and place on ice.

10. Sterilize the forceps by dipping in the ETOH, followed by flaming. With the head of the zebrafish pointing to the left, use the forceps to gently move the gut tissue over (towards you) until you locate the spleen. The spleen is about the size of the head of a pin and is the only dark red organ left in the body cavity. The bright green gall bladder is about the same size and will also be in this area.

Be careful when removing the gall bladder to grasp at the very top of the organ (where it is attached to the liver). The gall bladder can easily burst if grasped in the middle and pulled with the forceps. The spleen is also delicate and can rupture if it is pulled out with too much force.

11. Place all instruments in ETOH and prepare for removal of the brain. Remove the pins from the body and turn the fish over with the dorsal side up. Replace pins through the central back and the tip of the mouth. Swab the outside of the head with ETOH. Flame scalpel and place the tip of the scalpel against the back of the skull plate. Press down lightly and lift skull plate up from rest of fish. This step will take a few tries but it should come loose fairly easily. Removal of this plate exposes the brain tissue. Using sterile forceps, remove brain tissue and place in appropriate microfuge tube and place on ice.

If dissection of the brain is desired, always remove after dissecting the heart. This will help to limit contamination of the brain tissue with bacteria from the blood stream during dissection, resulting in elevated numbers being enumerated in the brain.

12. Lastly, if the injections were performed by IM, you may want to analyze the number of bacteria remaining in the muscle at the injection site. To do this, with the fish still in the dorsal side up position, locate the site of injection, which is on the upper left side of

the dorsal muscle. There should be a lesion at the site of injection. This lesion will vary in size and character depending on the streptococcal species used in each infection. Using the scalpel, cut a ~0.5cm^3 piece of tissue surrounding the injection site from the muscle. Carefully, swab the skin with ETOH and then using the sterile forceps peel back the skin from the muscle. Place the isolated muscle (without skin) in the appropriate microfuge tube on ice.

13. In a biosafety hood, homogenize the tissue in the PBS using the tissue homogenizer (Kontes hand-held cordless motor) fitted with a sterile, disposable pellet pestle, being careful not to create aerosols or lose any of the homogenate. Homogenize until a uniform liquid sample, which should take about 20–30 seconds depending on the organ. Use a new sterile pellet pestle for each organ. Place the tube back on ice after homogenization.

Homogenization should always be performed in biosafety hood with eye protection.

Enumeration of bacteria in tissues

14. To enumerate bacteria in each organ, make serial dilutions of each homogenate using sterile media. The number of dilutions will depend on the expected bacterial load and the time post infection. Plate the final two dilutions in triplicate on CNA plates and incubate in GASpak Jar (BBL) at 37°C.

CNA agar plates are selective for Gram-positive bacteria, inhibiting most Gram-negatives. This will inhibit any growth of bacteria that may have contaminated the organs (particularly the muscle) during isolation. Nevertheless, one should strive for sterile technique as much as possible to eliminate false positives.

Plates can also be incubated in a CO_2 incubator at 37°C, however, plates must be completely dry before placing in incubator as the excess humidity can cause problems with subsequent colony counting.

Cytospin preparations of infected tissues

Materials

1ml syringes

26 gauge needles

18 gauge needles

Cytofunnels Glass

microscope slides

Cytofuge (Shandon Elliot Cytospin 1 Centrifuge)

Protocol Hema-3 Stain system (Fisher)

15. To produce cytospin preparations from homogenized tissues for staining and microscopy, use the previously isolated organs (follow steps 1–12). Do not homogenize tissues that will be used for cytospins. Briefly vortex.

16. Pass homogenate through a 26-gauge needle on a 1 ml syringe. Repeat this step multiple times until most of the clumps of tissue are gone, placing homogenate back into original microfuge tube.

Multiple passes may be necessary to get all of the homogenate through the needle. Do not worry if part of an organ becomes lodged in the syringe. Passing the PBS and plunger over it several times will continue to dislodge single cells, which is the desired result instead of clumps of tissue. You may not be able to remove all of the tissue clumps.

17. Add 400ul of sterile PBS to the homogenate and vortex briefly. Place on ice.

18. For homogenates of muscles, add 200ul of PBS first, vortex briefly, then pass through an 18-gauge needle until fairly smooth, followed by passage through a 21-gauge needle.

The muscle tissue is much tougher than the other organs and will require passage through the two sizes of needle. There will still be some clumps of tissue left after the second passage.

19. Remove 100ul of this homogenate to a new microfuge tube and add 900 ul of sterile PBS and vortex briefly. Place on ice.

20. Load 300ul of each homogenate into a cytofunnel and place in cytofuge. Mark the slides with the strain, organ and time post infection. Spin for 3 minutes at 700 RPM.

To conserve the integrity of the cells, do not wait too long to perform cytospins after isolation of tissue. For best results, load funnels after they have been placed into cytofuge to avoid any spillage from the bottom of the funnel before centrifugation. Carefully remove slides and cytofunnels from cytofuge without smearing slide.

21. Immediately stain slides using the Protocol Hema-3 (Fisher) stain system. Dip the slide into each solution 10 times, followed by rinsing in 3 successive containers of tap water (3 dips/container). Allow the slides to air dry.

This stain is comparable to the Wright-Giemsa method of staining.

22. Once the slide is dry, cover stained area with mounting medium and a coverslip and view under light microscopy (see Figure 4 for cytospin of spleen homogenate).

Start at 400X and then move to 1000X with oil immersion. The best views will be at the outer 1/3 edge of the stained tissue where there will be more isolated cells and less clumping.

NOTE: Since tissues will start to degrade once the animal dies, it is best to limit the amount of time between dissection, homogenization, spinning and staining. Once the tissue is stained, it can be kept indefinitely. Therefore, it is best to limit the number of animals dissected at a time so that cytospins may be performed quickly after death of the animal. Ideally, multiple researchers working together can cut down the overall time.

BASIC PROTOCOL 3

HISTOLOGY OF INFECTED TISSUES

One of the advantages of using the zebrafish infectious disease model is the small size of the organism allows observation of all tissues from a longitudinal section on a single histology slide. This allows visualization of the infected tissues from site of injection to all organs of dissemination. This technique is useful for determining when and where the bacteria disseminate over time.

Fixation of Whole Zebrafish

Materials

Infected zebrafish (Basic protocol 1)

336 μg/ml Tris-buffered tricaine pH 7.0, for euthanizing fish

Plastic spoon

15 ml polypropylene conical tube

Sterile scalpel

Deitrich's fixative

Rocking platform shaker

1. At the desired time point after infection, place infected fish in Tricaine solution for 25 minutes to euthanize.

    Fish must be alive immediately prior to euthanization and fixation. Once the fish has died, decomposition of tissues occurs very rapidly and will not give an accurate representation of the infection course.

2. Remove the euthanized zebrafish from the Tricaine solution with a plastic spoon and place it on the dissecting board. Use a scalpel to remove the tail portion just behind anal fins.

    Removal of the tail portion allows better penetration of fixative to internal organs. Be careful not to cut any farther into body, beyond the anal fins, so as not to disrupt internal organs.

3. Place a single fish in a 15 ml conical tube with 10 ml of Deitrich's fixative. Secure the tube on a rocker and incubate in fixative with rocking at room temperature for 24 to 48 hours.

Caution: Dietrich's fixative contains acetic acid and formaldehyde. Proper precautions should be taken to limit exposure by preparing and pipetting the solution in a fume hood.

Embedding fixed tissues

Materials

Ethanol (ETOH) solutions (freshly made with ddH_2O)

50% ETOH

70% ETOH

95% ETOH

100% ETOH

Clearify (American Master Tech)

Histology tissue cassette (Fisher, #15-182-500E)

Tissue infiltration medium (Surgipath)

Formula R paraffin (Surgipath)

Waterbath at 60° to 64°C

Heat block at 60°C

Metal base mold for tissue cassette

4. Carefully drain Deitrich's fixative from conical tube into appropriately labeled chemical waste container for disposal, leaving fixed fish in tube.

5. Add 50% ETOH to the tube for 1 hour.

ETOH and Clearify steps should be done at room temperature with rocking.

6. Drain and add 70% ETOH for 1 hour.

7. Drain and add 95% ETOH for 1 hour.

8. Drain and add fresh 95% ETOH for 1 hour.

9. Drain and add 100% ETOH for at least 2 hours.

10. Drain and add Clearify overnight.

Clearify is a non-hazardous, non-flammable and non-carcinogenic substitute for Xylene solutions used in typical fixation procedures.

11. Drain and place fish section into a labeled tissue cassette. Add cassette to 60°C tissue infiltration medium (Surgipath) in a plastic screw cap container and incubate in a 60° to 64°C water bath for at least 1 hour.

12. Drain and add fresh infiltration medium and incubate in a 60° to 64°C water bath overnight.

13. Place fish in metal base mold with head facing to the right so as to allow the injection site (left side of fish) to be toward the outside of paraffin block, which is now the bottom of the base mold. Place cassette over the fish section with label to the right side.

14. Place the metal base mold containing the fish and tissue cassette on 60°C heat block to keep warm. Add melted 60°C Formula R paraffin (Surgipath) until cassette is covered. Gently remove base and cassette to ice block to cool. IMPORTANT NOTE: *Do not use infiltration medium at this step.*

15. After paraffin cools and hardens remove the cassette from the metal base. At this time, the embedded tissue sections can be stored indefinitely at room temperature for future sectioning.

Sectioning of paraffin-embedded tissues

Materials

Dawn dish detergent

Richardson Microtome (Model #HM 315)

Water bath for sections at 50° to 55°C

Glass microscope slides

16. Slides should be washed in acid-alcohol solution for at least 1 hour. Rinse in ddH_2O, 3 times for 5 minutes each rinse.

17. Soak paraffin blocks in a small container of water with 2 drops Dawn dish detergent and 2 drops of Downy to hydrate for 20 minutes. Keep container in ice bucket during hydration process.

While other dish detergents will also work, the authors have observed the best results with Dawn dish detergent.

18. Remove sections and dry with a paper towel just before cutting with microtome.

19. Cut block into 10-micron thick sections until you reach the area of interest, then section at 2 microns.

    If the block does not form ribbon sections properly or sections start to tear, resoak the block in the hydrating solution on ice for 10 to 15 minutes. In addition, using very sharp microtome blades is imperative for proper sectioning.

20. Float the cut paraffin ribbon sections in a water bath set at 50 to 55 C that has had gelatin added to the surface.

    The authors use powdered gelatin. Just a pinch (tip of a spatula) is needed to spread over the surface of the water.

21. Dip the acid-alcohol washed slide under the section floating on the water surface and pick up. Allow the slide to air-dry overnight.

    Take care to not bend or wrinkle the paraffin ribbon sections.

Deparaffinization and staining procedure

Materials

Clearify (American MasterTech)

Hematoxylin ETOH solutions:

100%, 95%, 80%, 70%, 50%, 30% ETOH

PBS

Eosin

Toluene

Glass staining dishes

Staining rack to hold slides

Set-up

22. Fill staining dishes with the following solutions:

    2 dishes with Clearify

    2 dishes with 100% ETOH

    1 dish with 95% ETOH

    1 dish with 70% ETOH

    1 dish with 50% ETOH

    1 dish with 30% ETOH

    1 dish with PBS

23. Add a staining rack with the dry paraffin slides to Clearify for 15 minutes.

    This step and the following steps should be done at room temperature.

24. Move the rack to the second dish with Clearify for 15 minutes.

25. Move the rack to the 100% ETOH dish for 2 minutes.

26. Move the rack to the second 100% ETOH dish for 2 minutes.

27. Move the rack to the 95%, 70%, 50%, and 30% ETOH dishes for 2 minutes each.

28. Finally, move the rack to the PBS dish for 5 minutes.

29. The slides should now be stained immediately with Hematoxylin & Eosin (H & E stain) or with fluorescent antibodies. The slides should not dry out before moving to staining procedure.

H & E staining

30. Fill the staining dishes with the following solutions:

    Hematoxylin

    ddH_2O

    80% ETOH

    Eosin

    2 dishes with 95% ETOH

    2 dishes with 100% ETOH

    2 dishes with Clearify

    1 dish with toluene

31. From the PBS dish, move the slide rack to Hematoxylin for 6 minutes.

    Hematoxylin needs to be freshly filtered using Whatman filter paper.

32. Carefully place slide rack into a container under slowly running tap water and dip rack 3–5 times into the container.

33. Place the rack in ddH_2O dish for 2 minutes.

34. Dip the slide rack into 80% ETOH for 10–15 dips.

35. Place in Eosin for 1 minute.

36. Dip into 95% ETOH for 10–15 dips.

37. Repeat in second 95% ETOH dish.

38. Dip into 100% ETOH for 10–15 dips.

39. Repeat in second 100% ETOH dish.

40. Place in Clearify for 1 minute.

41. Move to second Clearify dish for 1 minute.

42. Leave in fresh Clearify until ready to be cover slipped. Remove excess Clearify by carefully dipping in toluene. Dry slide and add mounting medium and cover slip (see Figure 5 for histology of infected tissues).

BASIC PROTOCOL 4

VIRULENCE ANALYSIS BY COMPETITIVE INDEX ASSAY

A competitive index assay allows the analysis of a mutant strain in direct comparison to the wild type strain within the same animal. This technique analyzes several parameters including: (1) the ability of the mutant to compete with the wild type strain for nutrients and colonization capacity in the same environment; (2) the ability of the mutant to survive in a host environment elicited by the wild type strain (host innate immune factors); (3) the ability

of the mutant to disseminate from the site of infection in comparison to the dissemination of the wild type strain; and (4) the ability of the mutant strain to be complemented by the presence of the wild type strain in the same environment. Since the infections are taking place in the same animal, any inherent factors of the individual host would be equal for both strains (host immunity, nutrition, age, overall health) instead of comparing single infection from two individual animals in which these factors may differ. In our experience, using the competitive assays in conjunction with single infections and LD50 analysis (see below) provides the greatest amount of information, as to the level of attenuation of a mutant.

Materials

Zebrafish

All materials needed for injections (see basic protocol 1)

Streptococcal bacterial cultures (see Support protocol 1 for preparation)

Agar plates with and without antibiotic

All dissection materials (see Basic protocol 2)

Microfuge tubes with 200ul PBS labeled with strain, organ and time point

Preparation

1. Set up work area for injections as described (Basic protocol 1)

2. Determine in advance the organs and time points to be analyzed.

3. Label in advance microfuge tubes with the strain, time point and organ to be collected. Fill each tube with 200ul sterile PBS.

4. Have agar plates prepared and warmed to room temperature. Label with strain, time point, organ and dilution to be plated.

> The number of serial dilutions will depend on the predicted amount of bacteria to be isolated from each organ at each specific time point. This can be estimated by doing small-scale preliminary experiments with the wild type strain alone.

Infections for competitive assays

5. Grow each culture individually as described in Support protocol 1 to mid-log phase and prepare cultures as for a typical injection, bringing each strain to 1×10^8 cfu/ml.

> One strain must have resistance to an antibiotic that is not in the other strain to allow selection by plating on agar with antibiotic. Usually the mutant has antibiotic resistance different from the wild type as a result of the mutagenesis technique.

6. Add 100ul of each strain (1×10^7 cfu) to a 1 ml microfuge tube and bring to 1 ml with 800ul sterile media. The two strains are now at a 1:1 ratio with a final total bacterial concentration of 2×10^7 cfu/ml in one microfuge tube.

7. Inject zebrafish as described in Basic protocol 1 with 10ul of the mixed bacterial culture, resulting in an infectious dose of 2×10^5 cfu.

8. Reserve an aliquot of this mixture on ice for subsequent serial dilution to determine the actual initial input ratio of mutant to wild type. The same serial dilutions need to be plated on agar (without antibiotic), to determine the concentration of total bacteria in the mixture, and on agar with antibiotic to determine the concentration of mutant bacteria in

the mixture. To determine the concentration of the wild type bacteria in the original injection, the concentration of mutant bacteria can be subtracted from the concentration of total bacteria determined from plating the mixture on agar without antibiotics. These numbers will be used for the input concentrations for the final competitive indices calculation.

9. Euthanize and dissect infected fish based on specific organs and time points desired.

For systemic infections, we typically remove the spleen, heart, brain and muscle (injection site), and examine at 24 hours post injection for competitive indices.

10. Dissect specific organs and homogenize tissue according to Basic protocol 2. Plate serial dilutions in triplicate on agar plates (each dilution with and without antibiotics) to determine the output ratio of the mutant strain to the wild type strain at each time point in each organ.

After each dissection, place tissue on ice to inhibit any additional bacterial growth. Regardless of time points and organs selected, it is better to keep the number of fish analyzed to 4 for each time point, unless multiple researchers are participating.

11. Six to 8 fish should be analyzed for each organ and time point for statistical analysis and significance.

12. The competitive index is calculated by dividing the output ratio (mutant/wild type) by the input ratio (mutant/wild type).

For example:

output cfu (mutant strain 3.62×10^3/wild type 2.47×10^5)=0.0147 input cfu (mutant strain 2.18×10^5/wild type 1.93×10^5)= 1.13

$0.0147/1.13 = 0.013 = 1.3 \times 10^{-2}$ or > 2 logs attenuation.

13. Final ratios of mutant to wild type that are less than 1 indicate a mutant is attenuated compared to the wild type for survival in vivo (see Figure 6 for competitive indices example).

BASIC PROTOCOL 5

QUANTITATIVE DETERMINATION OF VIRULENCE BY LETHAL DOSE 50

The lethal dose 50 (LD50) allows a quantitative measurement by which the virulence of one strain can be compared to another. The lethal dose 50 is the dose at which 50% of animals are killed. This analysis is particularly useful when comparing a mutant strain to the wild type parent strain or when comparing two serotypes of the same species that may have slightly different virulence profiles. The procedure requires a range of doses to be administered to groups of animals and therefore requires a greater than usual number of animals to be tested. Typically, 6 animals per group (dose) are infected. This experiment is repeated once, resulting in twelve animals per dose. If the LD50 of one strain is known, then usually 2 logs above and 2 logs below this number will give a good range in which to test a mutant.

Materials

Zebrafish (6–9 months)

All materials for injections (Basic protocol 1)

Bacterial cultures (Support protocol 1)

Materials for euthanization (Basic protocol 1)

Agar plates

1. Once the culture has reached mid log phase, bring all cultures to a concentration of 1 × 10^8 cfu/ml as described in Basic Protocol 1.

2. Dilute cultures with sterile media to the appropriate dose. Keep on ice until the culture is loaded into the syringe. Save an aliquot aside for subsequent plating to confirm the actual injected dose.

The LD50 for our streptococcal strains is approximately 10^3 to 10^4 cfu. We would therefore prepare inoculums of 10^2, 10^3, 10^4, 10^5, 10^6cfu per fish. Since the amount of injection is 10ul, this means that tubes with 10^4, 10^5, 10^6, 10^7 and 10^8 cfu/ml should be prepared.

3. Perform injections with 6 zebrafish per dose and place the fish in beakers labeled with strain, dose and date (Basic protocol 1) and place each beaker in 28°C incubator for observation.

4. Plate serial dilutions of the inoculums in duplicate and place in 37°C CO_2 incubator or in GasPak jars at 37°C.

5. Observe zebrafish over the course of 5 days, promptly removing dead zebrafish or removing and euthanizing moribund fish. Record all deaths with the date and time.

Removing dead or dying fish promptly is very important for preserving the water quality for the remaining fish. If the water becomes very cloudy, it should be changed with fresh water. However, overhandling of the fish during infections increases stress and could result in accelerated mortality and inaccurate resulting data.

NOTE: Please refer to local institutional IACUC guidelines for guidelines on mortality studies.

6. At the end of 5 days, euthanize the remaining zebrafish by placing in 336 ug/ml Tricaine for 25 minutes. Eliminate dead zebrafish according to institutional guidelines and regulations for disposal of infected animals.

7. Calculate LD50 according to Reed and Muench (Reed and Muench 1938).

BASIC PROTOCOL 6

ANALYSIS OF BACTERIAL DISSEMINATION

Both *S. agalactiae* and *S. iniae* cause systemic infections in the zebrafish, including dissemination to the brain. This protocol allows quantification of systemic spread of bacteria after either IM or IP injection. This procedure is most informative when multiple time points are analyzed. Bacterial spread to an organ can be determined by dissection and homogenization of the tissue followed by plating on selective media to enumerate bacteria. Not only does this procedure identify the tissues to which the bacteria can disseminate, but by comparing early time points to later time points, one can determine if the bacteria can proliferate in a certain organ or are cleared from the organ over time.

Materials

Infected zebrafish (Basic Protocol 1)

336 μg/ml Tris-buffered tricaine pH 7.0, for euthanizing fish

Plastic spoon

70% ETOH in 250ml beaker (~100ml)

Bunson burner

Personal protective equipment (lab coat, gloves and eye protection)

Sterile dissection instruments including:

Dissecting board (Styrofoam or cork cutting board)

Dissecting pins

Small pointed tip surgical scissors

Tissue forceps

Scalpel

37°C CO_2 incubator or 37°C incubator and GasPak Jars

Tissue homogenizer (Kontes cordless motor)

Disposable pellet pestles, sterile

Phosphate-buffered saline (PBS), sterile

1.5 ml microfuge tubes, sterile

Colistin-Nalidixic Acid (CNA) agar Petri plates

Note: While all personal protective equipment should be worn for all protocols in this chapter particular caution should be used during injections and dissections of the zebrafish.

1. Determine the desired time points and tissues to be analyzed and label 1.5 ml microfuge tubes with strain, time point, and organ and add 200 ul of sterile PBS to each tube.

For systemic infections, we typically remove the spleen, heart, brain and muscle (injection site), at 15, 30, 60 minutes and 2, 4, 8, 12 and 24 hours post injection, although fewer time points can be performed initially to determine if major differences are observed.

2. Bring previously prepared CNA agar plates to room temperature and label with strain, time point, organ and dilution.

Prepare enough CNA agar plates to plate the two final serial dilutions in triplicate. If the approximate number of bacteria in an organ is unknown (for a mutant strain for example), additional dilutions are necessary to avoid having plates with no bacterial growth. To conserve resources, small-scale experiments can be done in advance to estimate dissemination of particular mutant strains.

3. Follow Basic protocol 1 for injection of zebrafish by either IM or IP. Design a plan to stagger time points for injections, so that all injected fish are not ready to be dissected at the same time.

Since injections take much less time (seconds) than dissections (~5 minutes per fish depending on number of organs), leaving time between injections will make dissections and plating much more manageable.

4. Euthanize infected fish based on time points desired by placing in 336 ug/ml Tricaine solution for 25 minutes. Dissect specific organs, homogenize tissue and enumerate bacteria according to Basic protocol 2. An example of a dissemination graph is shown in Figure 7.

After each dissection, place tissue on ice to inhibit any additional bacterial growth. Again, staggering the injections over time will allow the euthanizations and dissections to also be staggered so that the time points are more manageable. Regardless of time points and organs selected, it is better to keep the number of fish analyzed to 4 for each time point, unless multiple researchers are participating.

SUPPORT PROTOCOL I

PREPARATION OF STREPTOCOCCAL CULTURES FOR ZEBRAFISH INFECTION

This protocol describes how to prepare *S. pyogenes* and *S. iniae* cultures for infection of zebrafish. Always use early to mid log phase cultures, as injection of stationary phase cultures results in early clearance by the host. Initially performing a growth curve with the strain of interest to determine the OD600 that correlates to log phase and determining an accurate bacterial concentration (cfu/ml) at this stage will save time and resources and make subsequent infections more reproducible. Once the correct dilution for injections has been made, keep all cultures on ice to inhibit additional growth. Aliquots of freezer stock (in ~30% glycerol) should be made to avoid multiple freeze/thaw cycles.

Materials

Todd Hewitt Broth + peptone (TP)

15ml sterile conical tubes with screw cap

Spectrophotometer

Microfuge tubes

Todd Hewett Agar plates (THY A)

1. Set up overnight cultures from a freezer stock in 10ml TP in a 15 ml conical tube with a screw cap without shaking.

   Add any appropriate antibiotics. Do not let the overnight culture grow more than 14 hours. Growth for more than 14 hours will result in an extended lag phase and poor culture growth overall.

2. Next morning vortex overnight culture well (~20 seconds) to re-suspend all bacteria. Dilute overnight culture 1:100 (1:50 for *S. pyogenes*) into a new 15ml tube with 10ml fresh TP including any appropriate antibiotics.

3. Grow at 37°C without shaking until mid-log phase or 1×10^8 cfu/ml.

   Check OD_{600} at 3 hours and then grow to a final OD_{600} over .300, but not over 0.500. For S. pyogenes HSC5, 1×10^8 cfu/ml = .30 and S. iniae 9117 = .225–.250 on a Beckman Coulter DU530 spectrophotometer. Adjust all cultures to the correct concentration by dilution.

4. Remove 1ml of the culture and place in a microfuge tube. Spin down in microcentrifuge, remove all supernatant, and re-suspend pellet in 1 ml fresh TP.

   This removes all secreted proteins found in the supernatant.

5. Make dilutions from this tube for the appropriate concentration for injection.

   Keep in mind that you will be injecting only 10ul into the zebrafish, meaning that 10ul of the 1×10^8 cfu/mltube will result in a 1×10^6 injection.

6. Make serial dilutions of all cultures for subsequent plating on THY A plates with appropriate antibiotics to confirm concentrations injected.

REAGENTS AND SOLUTIONS

Dietrich's Fixative

150 ml 95% ethanol

10 ml glacial acetic acid

185 ml 37% formaldehyde

155 ml H_20

Store indefinitely at room temperature

Todd-Hewitt Yeast Broth (THY B)

30 g Todd-Hewitt

2 g yeast extract

Bring to a final volume of 1000 ml. Stir until completely dissolved. Dispense 100 ml into bottles and autoclave. Store at room temperature.

For agar plates (THY A): Add 14 g high quality agar (we use Difco Bacto-agar) before autoclaving. Autoclave. Cool to 55°C. Add appropriate filter sterilized antibiotics if needed. Pour ~30 ml into 100 × 15 mm plates. Store at 4°C. Plates without antibiotics can be stored up to 6 months. Plates with antibiotics should be used within 30 days or less (depending on antibiotic).

Todd-Hewitt Yeast Peptone Broth (TP)

30 g Todd-Hewitt

2 g yeast extract

20 g Protease Peptone #3

Bring to a final volume of 1000 ml. Stir until completely dissolved. Dispense 100 ml into bottles and autoclave. Store at room temperature.

For agar plates: Add 14 g high quality agar before autoclaving. Autoclave. Cool To 55°C. Add appropriate filter sterilized antibiotics if needed. Pour ~30 ml into 100 × 15 mm plates. Store at 4°C. Plates without antibiotics can be stored up to 6 months. Plates with antibiotics should be used within 30 days or less (depending on antibiotic).

Columbia Colistin Nalidixic Acid agar (CNA)

43 g CNA

Bring to a final volume of 1000 ml. Stir well (will not completely dissolve before autoclaving). Autoclave. Cool to 55°C. Add appropriate filter sterilized antibiotics if needed. Pour ~30 ml into 100 × 15 mm plates. Store at 4°C.

Tricaine Stock Solution

400 mg tricaine

97.9 ml ddH_20

2.1 ml 1M Tris (pH 9)

Note: It is very important to use pH 9 Tris for the stock solution and then adjust to pH 7 after adding the water and tricaine.

Adjust pH to ~7. Filter sterilize and store up to one month at 4°C.

For anesthetizing zebrafish: ~168 ug/ml:

Add 4.2 ml Tricaine stock solution to 96 ml of sterile tank water. Make fresh solution each time.

For euthanizing zebrafish: ~336 ug/ml:

Add 8.4 ml Tricaine stock solution to 92 ml of sterile tank water. Make fresh solution each time.

Acid-Alcohol Wash solution for slides

8 ml HCL

added to 1 liter 70% ETOH

Slides should be washed in acid-alcohol solution for at least 1 hour. Rinse in ddH_2O, 3 times for 5 minutes each rinse.

Aquarium Tank Water

Tap water

0.5 ml/gallon of Amquel

Water for infections

ddH_20

60 mg/L Instant Ocean

Autoclave. Store at room temperature indefinitely.

COMMENTARY

Background Information

The zebrafish has been shown to be a valuable animal model for infectious disease by multiple groups, utilizing a variety of bacterial and viral pathogens (Phelps 2005). Not only has the zebrafish been infected with natural fish pathogens, such as *Streptococcus iniae*, but also with human clinical isolates such as *Streptococcus pyogenes* and *Streptococcus agalactiae* (Neely, Pfeifer et al. 2002; Phelps 2005). Such studies illustrate the utility of the zebrafish animal model of infectious disease for examination of multiple streptococcal pathogens and distinctly different disease states. More importantly, the pathology associated with streptococcal infections observed in the zebrafish resembles that seen in human cases. The zebrafish model for necrotizing fasciitis exhibits little to no inflammatory cell infiltration and high bacterial load, comparable to that observed in both human and other animal model studies (Cockerill, Thompson et al. 1998; Hidalgo-Grass, Dan-Goor et al. 2004; Thulin, Johansson et al. 2006). Similarly, *Streptococcus iniae* has been shown to cause invasive infections leading to meningitis and endocarditis in both fish and humans (Weinstein, Low et al. 1996; Weinstein, Litt et al. 1997; Miller and Neely 2004).

S. pyogenes is a Gram positive, β-hemolytic strict human pathogen that is responsible for an incredible variety of diseases, which include pharyngitis, scarlet fever, impetigo, cellulitis, streptococcal toxic shock syndrome (STSS), necrotizing fasciitis, and septicemia (Bisno and Stevens 1996; Cunningham 2000). This streptococcal pathogen remains a significant cause of morbidity and mortality in the United States and worldwide. Multiple factors come into play in determining the course of infection including a significant array of virulence factors produced by *S. pyogenes* as well as the opposing action of the host immune response activated by the presence of the pathogen. Developmental studies have shown that the

zebrafish exhibits both innate and adaptive immune responses (Phelps 2005). The interaction of *S. pyogenes* with host macrophages in the zebrafish parallels what has been observed in human cases of invasive disease (Thulin, Johansson et al. 2006; Phelps and Neely 2007).

Streptococcus iniae, a zoonotic pathogen, is also a significant Gram-positive pathogen that is skilled at evading the host immune response. Capsule has been demonstrated to have a substantial role in evading and modulating the host immune response, and in turn in causing severe systemic disease (Buchanan, Stannard et al. 2005; Miller and Neely 2005; Locke, Colvin et al. 2007; Lowe, Miller et al. 2007), as well as an M-like protein and a β-hemolysin (Locke, Colvin et al. 2007; Locke, Aziz et al. 2008). *S. iniae* is highly adapted for causing naturally occurring disease in fish, and yet this organism has a wide host range capable of infecting humans and a murine model of systemic disease (Weinstein, Low et al. 1996; Weinstein, Litt et al. 1997).

Critical Parameters and Troubleshooting

Problems associated with initial zebrafish infections can be avoided by beginning with smaller scaled experiments. Well-planed experiments will lead to more consistent results. Experiments should always include a control set of fish injected with sterile media alone. This group of media fish controls for the anesthetization and injection process, as well as for the general health of the group of fish being used in an experiment. In addition, initial experiments should be conducted to determine the optimal infectious dose for subsequent experiments. Ideally, the lowest possible inoculum concentration that is 80%–100% lethal should be used. In the preparation of the inoculum, the bacteria should be washed and resuspended in fresh media prior to injection. This washing step removes excess secreted factors that may impact the infection. When evaluating mutant strains, growth kinetics should be examined and compared to the wild type strain in vitro prior to infecting animals. Growth curves generated from plating bacteria over a time course can be used to determine at what optical density the culture has reached mid-log phase, 1×10^8 cfu/ml. Mutants that exhibit inhibition of growth in vitro can be further evaluated using chemically defined medium to determine the growth deficiency. In vivo experiments examining these mutants may be evaluated differently than from other mutants.

Great care should be taken to not cause undue stress to the animals at any time before or during the course of an experiment. Tricaine solution used for anesthetizing the fish should be prepared as described and at the appropriate concentration. If this solution is not prepared correctly (i.e., not using Tris pH 9) the fish may bleed from the gills, may have difficulty recovering from the treatment, or may even die. Any additional stress on the animal can lead to an altered outcome of the infection. Only small numbers of fish should be removed from the main aquarium at a time for injections, so that fish are not kept at room temperature for an extend amount of time. Each fish should be injected and placed back into water as quickly as possible while still maintaining the safety of the researcher performing the injections. Groups of no more than 4–6 fish should be housed in the 400ml beakers, and because fish are not fed during the course of the infection the experiment should not extend beyond five days post injection. Fish that die during the infection should be removed as quickly as possible to keep from compromising the water quality for the remaining fish. Fish with aberrant swimming behavior or those that are swimming around the top of the beaker and do not exhibit a startle response are close to death and should be euthanized. In addition, any fish with a large necrotic lesion (extending over the side and back of the fish) is unlikely to recover and should also be euthanized. If the beaker water holding the infected fish becomes overly cloudy during the course of the 5 days, it should be replaced with fresh tank water to prevent loss of additional fish by lack of sufficient oxygen.

The bacteria should be in log phase growth for injections. Cultures of stationary phase bacteria are most often cleared early in the infection by the host. This requires setting up a fresh culture from freezer stocks the night before. For greater reproducibility, aliquots of 100ul of frozen glycerol stocks prepared for the strains used most often is advisable so that freezer stocks are not put through multiple freeze/thaw cycles. In this way, an aliquot can be used once and then discarded, safe guarding the original freezer stock. For accurate dilutions and CFU counts, always vortex cultures well before making serial dilutions for plating or injection to break up long chains into a homogeneous mixture. If a mutant strain forms long chains compared to the parent strain, then the resulting cfu will be artificially low if the culture is not vortexed, as each chain will form a single colony. However, this can be avoided if all cultures are vortexed before dilution as most chains are broken up by vigorous vortexing.

Anticipated results

IM and IP injections—Signs of illness should be monitored closely during the course of infection. Under normal circumstances IM injections of *S. pyogenes* at a concentration of 1×10^5 cfu/ml (~approximately 100 times the LD50) results in a lethal infection with 100% death occurring approximately 48 hours post injection. At the 24 hour point, many if not all of, the fish infected with the wild type strain of *S. pyogenes* should have a hypo-pigmented lesion at the site of injection. IM infections with *S. pyogenes* have an LD_{50} ~3×10^3 cfu/ml. IP infections have a slightly higher LD_{50} of 1×10^4 cfu/ml. *S. iniae* has an LD_{50} of ~1×10^3 cfu/ml for both IM and IP injections. At a concentration of 1×10^5 cfu/ml, *S. iniae* results in a lethal infection with death occurring at roughly 48–72 hours. An IM injection with *S. iniae* will only occasionally result in a noticeable lesion at the injection site.

Cytospins—Zebrafish injected with the wild type strain (IM) are euthanized and the spleens are dissected and gently homogenized. The homogenate is added to a cytospin column, which during a centrifugation step applied a single layer of cells to a microscope slide. *S. pyogenes* infected zebrafish show evidence of a substantial bacterial load in the spleen at 12–20 hours post injection, and while some chains of cocci are found extracellular, the majority of the bacteria are localized intracellular within macrophages at late time points. *S. iniae* infection and dissemination to the spleen results in high bacteria load of intracellular bacteria within macrophages (Figure 4).

Histological analysis of infected tissues—The pathology of an IM infection with a wild type strain of *S. pyogenes* is characterized by extensive necrosis of the dorsal muscle with significant bacterial load. The bacteria aggregate in the fascial planes of the muscle tissue, and often in regions of significant necrosis, microcolony formation can be observed. The infection is notably lacking infiltration of inflammatory cells. The pathology associated with *S. iniae* IM infections is distinct from that of *S. pyogenes*, in that the level of tissue destruction is less and there is a great deal of infiltration of inflammatory cells (Figure 5).

Dissemination—The inflammatory response observed in histological analysis does not inhibit the capability of *S. iniae* from readily disseminating to multiple organs, including the heart, spleen, liver, gall bladder and brain as early as 15–60 minutes post injection (Figure 7). Conversely, significant dissemination with *S. pyogenes* is not observed until much later time points, approximately 8–12 hours post injection, and is observed mainly in the heart and spleen.

Time Considerations

Dilution of an overnight culture into fresh media is required for infections to allow the bacteria to grow to log phase as described in the Support Protocol. This should take about 3

hours. During this time, all of the injection instruments/equipment, beakers, microfuge tubes for dilutions and plating can be set up in preparation for the injections. This includes labeling and putting water into the beakers, setting up microfuge tubes for serial dilutions, getting out agar plates for plating, etc. The set-up should take approximately an hour depending on the number of strains that will be injected. The actual time for injecting the zebrafish will depend on the number of fish to be injected, but ~ 5 minutes per group of 6 fish is a good estimate. After injections are completed, dilution and plating of the strains injected will take about 5 minutes per strain.

As mentioned earlier, dissections will take about 5 minutes per fish, depending on the number of tissues to be harvested. Microfuge tubes with PBS should be set-up and labeled before dissections begin. Remember to include additional time for euthanization. Homogenization for plating takes about 1 minute per microfuge tube, and since the integrity of the tissues is not an issue when analyzing dissemination, tubes may be placed on ice until ready for plating. Homogenization for cytospins (using syringe and needle) takes a bit longer, approximately 3 minutes per organ. Setting up cytofunnels and completing the centrifugation will take ~10 minutes, followed by ~3 minutes per slide for staining.

Literature Cited

Bisno AL, Stevens DL. Streptococcal infections of skin and soft tissues. New Engl J Med. 1996; 334:240–245. [PubMed: 8532002]

Buchanan JT, Stannard JA, Lauth X, Ostland VE, Powell HC, Westerman ME, Nizet V. *Streptococcus iniae* phosphoglucomutase is a virulence factor and a target for vaccine development. Infect Immun. 2005; 73(10):6935–44. [PubMed: 16177373]

Cockerill FR 3rd, Thompson RL, Musser JM, Schlievert PM, Talbot J, Holley KE, Harmsen WS, Ilstrup DM, Kohner PC, Kim MH, Frankfort B, Manahan JM, Steckelberg JM, Roberson F, Wilson WR. Molecular, serological, and clinical features of 16 consecutive cases of invasive streptococcal disease. Southeastern Minnesota Streptococcal Working Group. Clin Infect Dis. 1998; 26(6):1448–58. [PubMed: 9636878]

Cosma CL, Swaim LE, Volkman H, Ramakrishnan L, Davis JM. Zebrafish and frog models of *Mycobacterium marinum* infection. Curr Protoc Microbiol. 2006; Chapter 10(Unit 10B):2. [PubMed: 18770575]

Cunningham MW. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev. 2000; 13(3): 470–511. [PubMed: 10885988]

Hidalgo-Grass C, Dan-Goor M, Maly A, Eran Y, Kwinn LA, Nizet V, Ravins M, Jaffe J, Peyser A, Moses AE, Hanski E. Effect of a bacterial pheromone peptide on host chemokine degradation in group A streptococcal necrotising soft-tissue infections. Lancet. 2004; 363(9410):696–703. [PubMed: 15001327]

Locke JB, Aziz RK, Vicknair MR, Nizet V, Buchanan JT. *Streptococcus iniae* M-like protein contributes to virulence in fish and is a target for live attenuated vaccine development. PLoS ONE. 2008; 3(7):e2824. [PubMed: 18665241]

Locke JB, Colvin KM, Datta AK, Patel SK, Naidu NN, Neely MN, Nizet V, Buchanan JT. *Streptococcus iniae* capsule impairs phagocytic clearance and contributes to virulence in fish. J Bacteriol. 2007; 189(4):1279–87. [PubMed: 17098893]

Locke JB, Colvin KM, Varki N, Vicknair MR, Nizet V, Buchanan JT. *Streptococcus iniae* beta-hemolysin streptolysin S is a virulence factor in fish infection. Dis Aquat Organ. 2007; 76(1):17–26. [PubMed: 17718161]

Lowe BA, Miller JD, Neely MN. Analysis of the polysaccharide capsule of the systemic pathogen *Streptococcus iniae* and its implications in virulence. Infect Immun. 2007; 75(3):1255–64. [PubMed: 17194809]

Miller JD, Neely MN. Zebrafish as a model host for streptococcal pathogenesis. Acta Trop. 2004; 91(1):53–68. [PubMed: 15158689]

Miller JD, Neely MN. Large-scale screen highlights the importance of capsule for virulence in the zoonotic pathogen *Streptococcus iniae*. Infect Immun. 2005; 73(2):921–34. [PubMed: 15664934]

Neely M, Pfeifer J, Caparon MG. Streptococcus-zebrafish model of bacterial pathogenesis. Infect Immun. 2002; 70(7):3904–3914. [PubMed: 12065534]

Phelps HA, Neely MN. SalY of the Streptococcus pyogenes lantibiotic locus is required for full virulence and intracellular survival in macrophages. Infect Immun. 2007; 75(9):4541–51. [PubMed: 17576754]

Phelps HA, Neely MN. Evolution of the Zebrafish Model: From Development to Immunity and Infectious Disease. Zebrafish. 2005; 2(2):87–103. [PubMed: 18248169]

Reed LJ, Muench H. A simple method of estimation of fifty percect endpoints. Am J Hyg. 1938; 27:493–497.

Thulin P, Johansson L, Low DE, Gan BS, Kotb M, McGeer A, Norrby-Teglund A. Viable Group A Streptococci in Macrophages during Acute Soft Tissue Infection. PLoS Med. 2006; 3(3):e53. [PubMed: 16401174]

Weinstein MR, Litt M, Kertesz DA, Wyper P, Rose D, Coulter M, McGeer A, Facklam R, Ostach C, Willey B, Borczyk A, Low DE. Invasive infections due to a fish pathogen, *Streptococcus iniae*. New Engl J Med. 1997; 337(9):589–594. [PubMed: 9271480]

Weinstein MR, Low DE, McGeer A, et al. Invasive infection due to Streptococcus iniae: a new or previously unrecognized disease. CCDR. 1996; 22:129–132.

Weinstein MR, Low DE, McGeer A, Willey B, Rose D, Coulter M, Wyper P, Borczyk A, Lovgren M. Invasive infection with *Streptococcus iniae*-Ontario, 1995–1996. MMWR Weekly. 1996

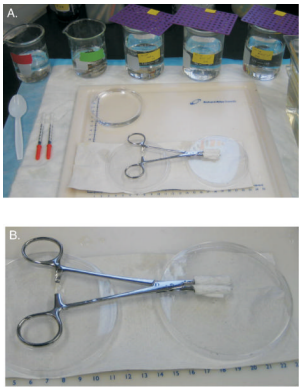
Westerfield, M. Guide for the laboratory use of zebrafish (Danio rerio). 4. Eugene: Univ. of Oregon Press; 2000. The zebrafish book.

Key References

Neely M, Pfeifer J, Caparon MG. Streptococcus-zebrafish model of bacterial pathogenesis. Infect Immun. 2002; 70(7):3904–3914. This manuscript describes the development of the zebrafish infectious disease model with infection procedures, typical infection parameters, and course of infection. [PubMed: 12065534]

Phelps HA, Neely MN. Evolution of the Zebrafish Model: From Development to Immunity and Infectious Disease. Zebrafish. 2005; 2(2):87–103. This is a comprehensive review of bacterial species used to infect zebrafish and the zebrafish immune response to infection. [PubMed: 18248169]

Westerfield, M. The zebrafish book. Guide for the laboratory use of zebrafish (Danio rerio); 4; Eugene: Univ. of Oregon Press; 2000. This describes basic protocols for zebrafish breeding, maintenance and analysis. Available online at http://www.zfin.org



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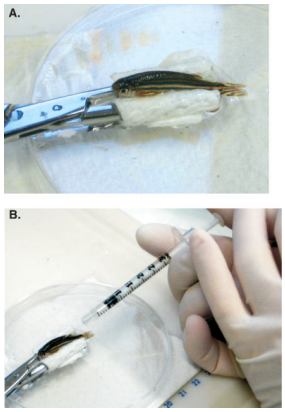

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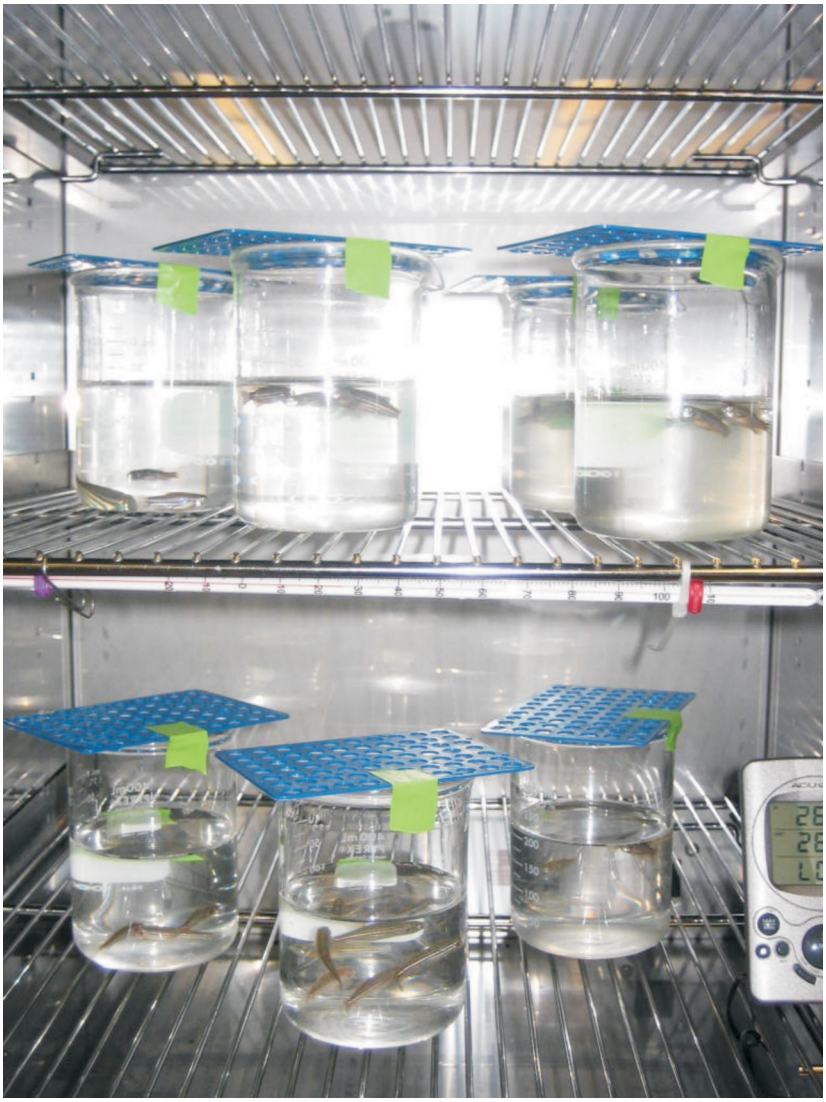

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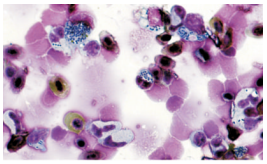

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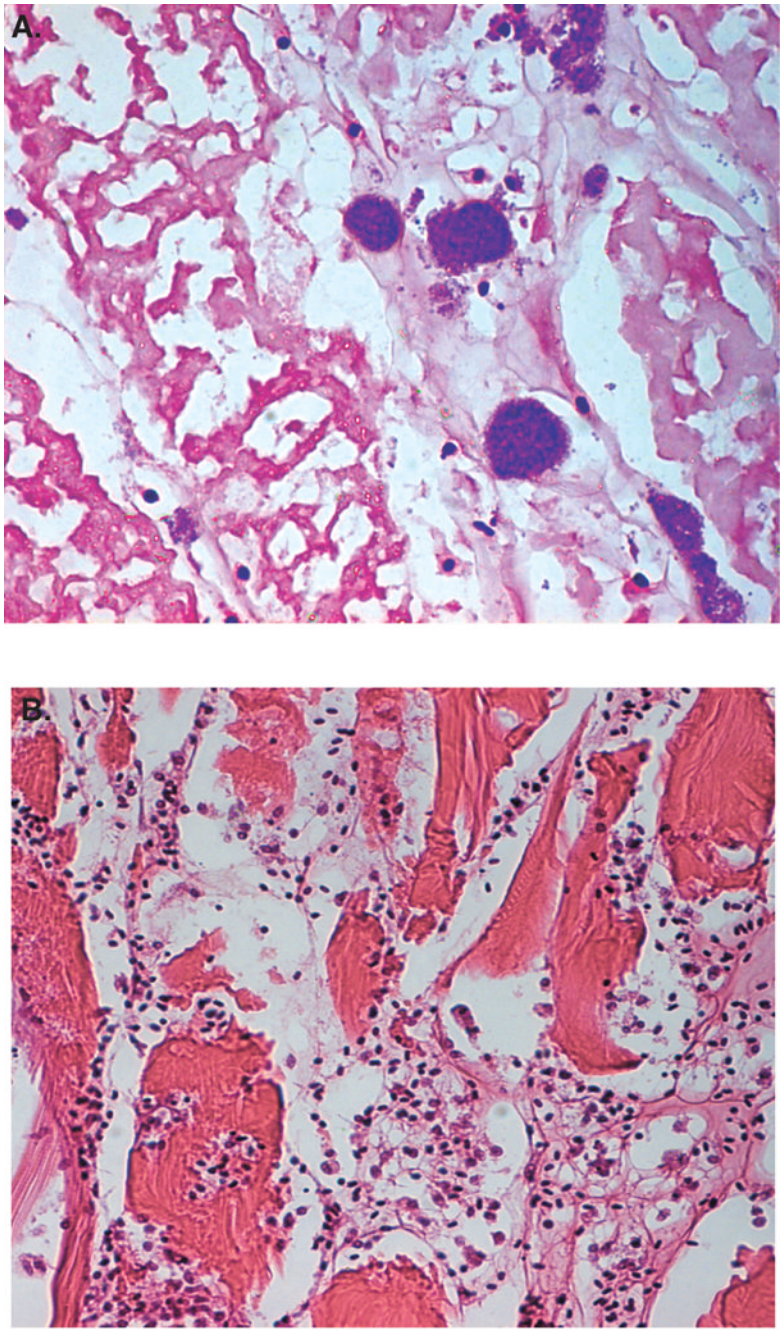

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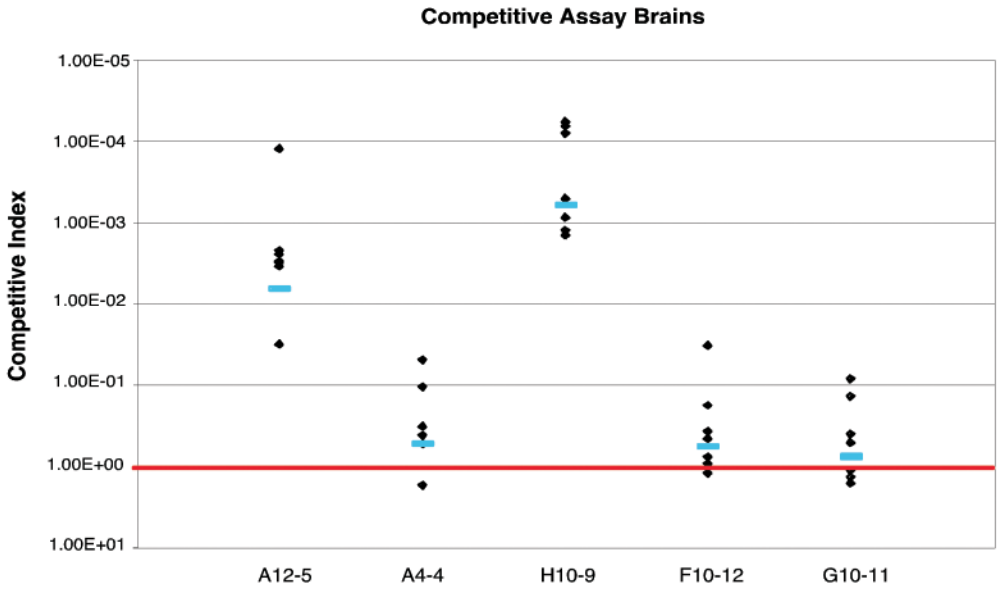

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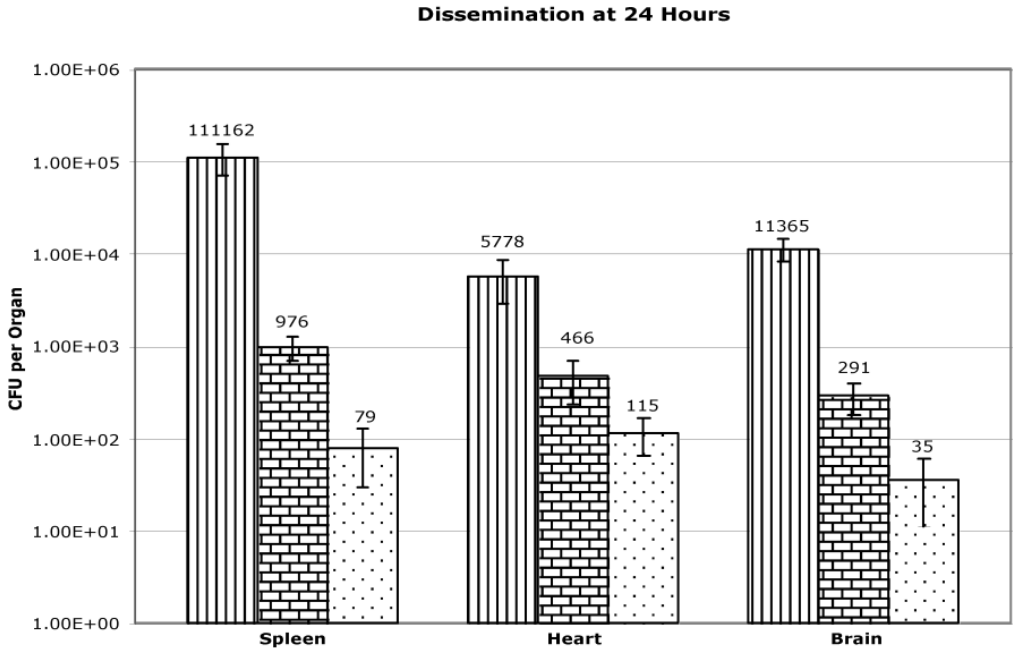

Figure 7.