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Protocol for infection of *Hymenochirus boettgeri* with *Batrachochytrium dendrobatidis* (Bd) and Ranavirus (Rv)

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We use this protocol and it's working

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Abstract

This protocol outlines the procedures for exposing *H. boettgeri* specimens to the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (Bd) or the Ranavirus Frog Virus 3 (Rv FV3), or both, for co-infection studies. These experiments are necessary to better understand the impacts of these lethal diseases on wildlife and the potential outcome of their interactions on hosts in a controlled laboratory setting. To ensure the safety and accuracy of the experimental exposures, the protocol provides step-by-step instructions and guidelines for handling these pathogens and administering the exposures. All procedures described here were performed in a temperature-controlled room set at 20°C and 13:11 day-night cycle.





Guidelines

Conclusion:

This protocol provides a guide for conducting exposures of *H. boettgeri* specimens to Bd, FV3, or both pathogens, ensuring the welfare of the amphibians and the accuracy of the experiments. Since all FV3 inoculation methods were effective in *H. boettgeri*, we recommend using the 'water bath' method as it is the least invasive approach. By following these detailed procedures and utilizing the specified materials, researchers can contribute to the advancement of our understanding of amphibian diseases and co-infections, ultimately aiding in their conservation and protection.



Materials

- H. boettgeri specimens
- 50 mL sterile Falcon tubes
- nitrile gloves
- Reverse osmosis (RO) water
- Synthetic sea salt mixture (Instant Ocean® Sea Salt, Blacksburg, VA, USA)
- Infectious Bd zoospores stored in sterile water from a pure culture
- Infectious FV3 stored in ABPS (Amphibian Balanced Physiological Solution) — a mixture of 100 mL of 1X PBS (phosphate-buffered saline, Invitrogen) and 25 mL of sterile Milli-Q water to achieve amphibian osmolarity (225 ± 5 mOsm/L)
- 30-gauge 0.3 mL 8 mm sterile syringe
- P100 pipetman
- P1000 pipetman
- 100 μ L pipetman tips
- 1000 μ L pipetman tips
- 0.73mm Tortex Flex standard guitar picks (reusable after bleaching)
- 1% tryptone media (1% agar) in 10 cm Petri dishes
- Bemis Parafilm™
- Corning® 40 μ m cell strainer (431750) – for use with 50 mL Falcon tubes
- Baby hamster kidney-21 cells (BHK-21; ATCC no. CCL-10)
-  DMEM, high glucose, pyruvate **Thermo Fisher Catalog #11995073**
-  Fetal Bovine Serum **Thermo Fisher Catalog #10437010**
-  Penicillin/Streptomycin **Invitrogen - Thermo Fisher Catalog #15140-122**
-  Cell culture flasks with standard or filter caps, CELLSTAR® **VWR International (Avantor) Catalog #82050-856**


Troubleshooting






Production of pathogens

1w 0d 1h

1 Preparation of Bd inoculum



1.1 Inoculate several 10 cm Petri dishes containing 1% tryptone media with Bd liquid culture by adding  500 μL with a 1000 pipetman. Usually, one plate per 2 frogs is sufficient. Seal all Petri dishes with parafilm.

1.2 Inoculate Bd onto new plates from either an actively growing liquid or Petri dish culture to start an inoculum preparation. For liquid inoculation, add ~  100 μL of an active growing culture and finish by adding  300 μL of sterile water. For inoculum from Petri dish cultures, cut 1 cm x 1cm square containing Bd with a lab sterile spatula, then gently rub this onto a new 1% tryptone Petri dish, and finish by adding  300 μL of sterile water. Seal all Petri dishes with parafilm.

1.3 Keep plates at  20 $^{\circ}\text{C}$ and check daily for maximum zoospore production.

Note

This typically occurs in 4-5 days if the culture is inoculated with fresh Bd material (i.e., abundant zoospores actively swimming around).

1.4 Add  2 mL of sterile water to the Bd culture plates. Wait for  01:00:00, then gently collect the water containing Bd zoospores by pipetting.




1.5 Transfer all the liquid into a 50 mL sterile Falcon tube. If any clumps of zoosporangia are noted, filter the inoculum using a 40 μL cell strainer (431750).

1.6 Calculate zoospore concentration using either a hemocytometer or Coulter counter. If necessary, dilute by adding sterile water.

1.7 All procedures describe here should be conducted in a sterile condition, ideally in a biological safety cabinet.

2 Cell line and FV3 high titer stocks production



- 2.1 Maintain baby hamster kidney-21 cells (BHK-21; ATCC no. CCL-10) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL), in a sterile cell culture flask with a vented cap, with 5% CO₂ at  37 °C .
- 2.2 Produce FV3 ([Granoff et al., 1965](#); ATCCVR-569 strain) stocks by a single infection passage on BHK-21 cells, culture for  168:00:00 at  30 °C in 5% CO₂ (in 250mL cell culture vented flasks). 1w
- 2.3 Purify FV3 virus particles by ultracentrifugation on a 30% sucrose gradient and quantification by plaque assay on BHK-21 monolayers in 6-well plates under an overlay of 1% methylcellulose ([Morales et al., 2010](#)).
- 2.4 Aspirate the overlay media and stain the cells for 10 min with 1% crystal violet in 20% ethanol before counting.

FV3 or Bd Exposure

- 3 Water Bath for FV3 and/or Bd
- 3.1 Begin by adding at least 3 mL of conditioned water, as described in the "Housing and Care for *Hymenochirus boettgeri*", containing the desired concentration of FV3, Bd, or both, to a 50 mL Falcon tube (Figure 1A).



Figure 1. Visualization of pathogen (FV3 and Bd) inoculation methods of *H. boettgeri*. Water bath of an adult *H. boettgeri* in a 50 mL Falcon tube filled with 5 mL of Bd noculum (A).

- 3.2 Change sterile gloves for each animal handled. Place an individual adult amphibian into each Falcon tube and keep them in the water bath for 2-3 hours.
- 3.3 After this exposure period, transfer the animals back to their permanent tanks.
- 4 Buccal Inoculation for FV3 Exposure
 - 4.1 With sterile gloves for each animal, hold the amphibian by its legs with one finger supporting its back and head (Figure 1B).

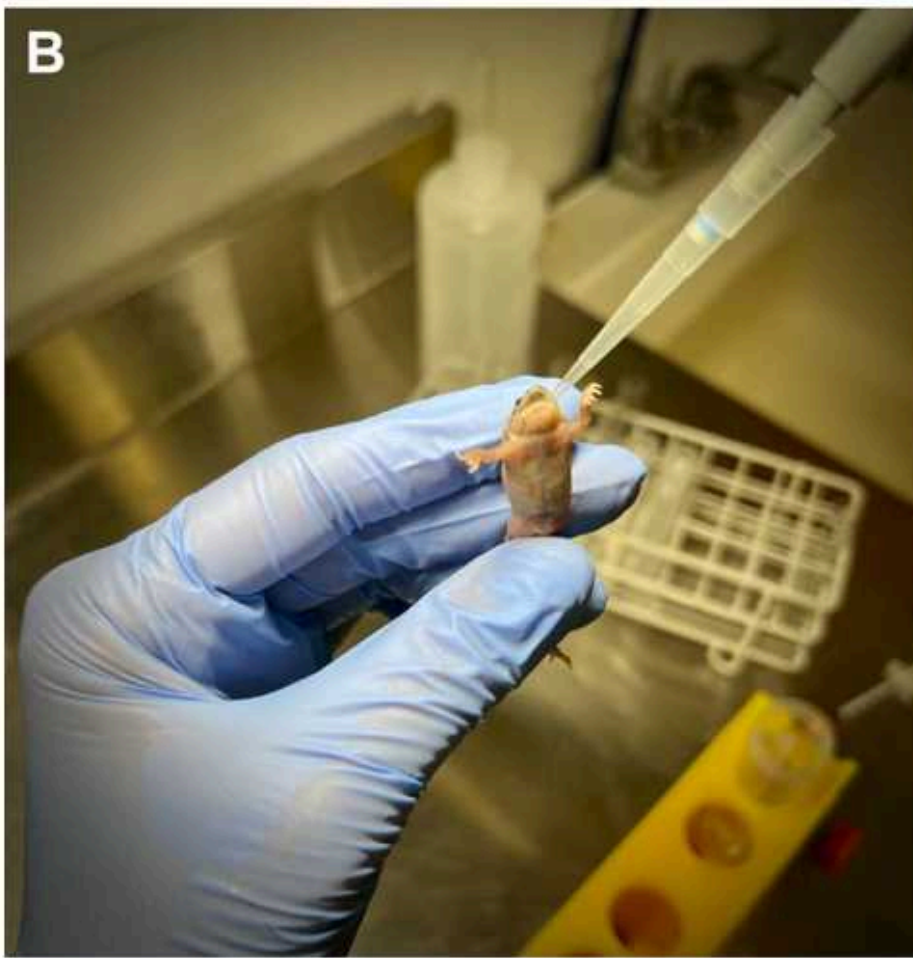


Figure 1. Visualization of pathogen (FV3 and Bd) inoculation methods of *H. boettgeri*. Water bath of an adult *H. boettgeri* in a 50 mL Falcon tube filled with 5 mL of Bd inoculum (A). Oral inoculation of *H. boettgeri* with FV3 (B)

- 4.2 Open its mouth by gently inserting and twisting a sterilized guitar pick, or similar device, in the side of the mouth opening. Using a pipetman and 100 μ L pipette tips, slowly inject up to 40 μ L of the desired concentration of FV3 inoculum into the amphibian's mouth.
- 4.3 Keep the amphibian still with the pipette tip inside its mouth for 5 seconds to prevent regurgitation.
- 4.4 Place the animal back in its permanent tank and repeat the procedure, if necessary to achieve total exposure dose, after at least 30 minutes to minimize stress on the animal.
- 5 FV3 Exposure by Intraperitoneal Injection

- 5.1 Using sterile gloves for each animal, hold the amphibian by its legs with its belly exposed and turned upward, supporting its back (Figure 1C). The frog should be immobilized. We sterilize the area of injection using 75% EtOH first, then inject FV3.

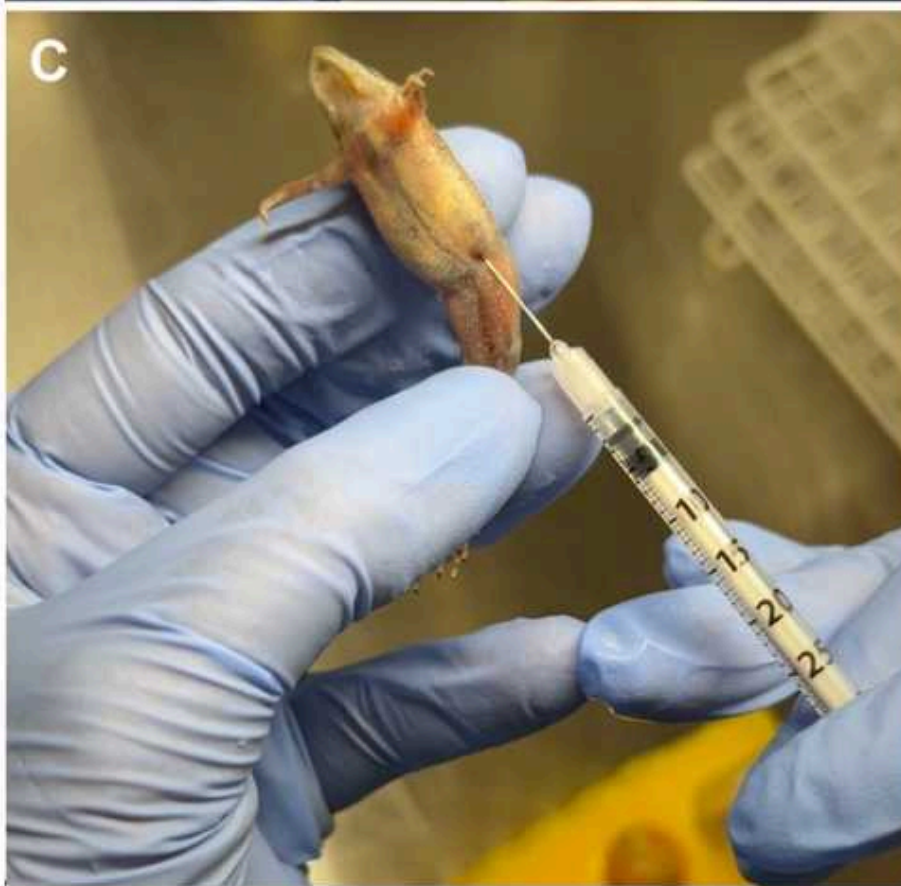


Figure 1. Visualization of pathogen (FV3 and Bd) inoculation methods of *H. boettgeri*. Water bath of an adult *H. boettgeri* in a 50 mL Falcon tube filled with 5 mL of Bd inoculum (A). Oral (B) and intraperitoneal (C) inoculation of *H. boettgeri* with FV3.

- 5.2 Carefully insert a needle at a shallow angle just anterior to and above the thigh, aiming toward the head (45-degree angle).
- 5.3 Gently push the syringe until you feel a slight 'pop,' indicating the needle is inside the peritoneal cavity, and then slowly release the liquid, injecting up to 60 μ L. Place the animal back in its permanent tank.
- 6 Dose responses
- 6.1 We have observed mortality of *H. boettgeri* adults given the following dose ranges: 104 to 107 Bd zoospores per mL (varying with Bd strain), 106 to 107 FV3 plaque forming units



(PFU) per mL in water bath exposure, 106 to 107 total PFUs in buccal inoculations, and 104 to 106 total PFUs in intraperitoneal injections.

6.2 Overall, for both pathogens and all exposure methods, higher doses lead to faster animal mortality and reduced chances of recovery from infection.

6.3 However, we acknowledge that susceptibility may vary not only with the dose but also with pathogen genotype and host characteristics, such as microbiome composition and prior infections. Therefore, we recommend conducting a pilot study to determine the optimal dose for both pathogens.

Protocol references

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