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# Systematic approach to isolating *Batrachochytrium dendrobatidis*

Anthony W. Waddle\*, Marlai Sai, Joshua E. Levy, Ghazal Rezaei,  
Frank van Breukelen, Jef R. Jaeger

School of Life Sciences, University of Nevada, Las Vegas, 4505 S. Maryland Parkway, Las Vegas, NV 89154, USA

**ABSTRACT:** We developed a protocol for isolating the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) from anurans. We sampled skin tissues from 2 common treefrogs, *Pseudacris regilla* and *P. triseriata*, collected from populations with high infection prevalence. We sampled tissues from 3 anatomical ventral regions (thigh, abdomen, and foot) where the pathogen is thought to concentrate. To mitigate potential bacterial contamination, we used a unique combination of 4 antibiotics. We quantified infections on frogs as zoospore equivalents (ZE) using a swabbing approach combined with quantitative real-time polymerase chain reaction. We isolated *Bd* from 68.9% of frogs sampled from both species. Contamination was low (9.7% of all plates), with most contamination presumed to be fungal. We found positive correlations between successful isolation attempts and infection intensity. Our levels of isolation success were 74% for *P. triseriata* and 100% for *P. regilla* once *Bd* detection intensities reached  $\geq 40$  ZE. Of the 3 anatomical regions sampled in both species, we had significantly more success isolating *Bd* from foot tissue. Our results support published recommendations to focus sampling for *Bd* infection on feet, particularly webbing.

**KEY WORDS:** Amphibian · Chytridiomycosis · Fungus · Isolation · Protocol

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## INTRODUCTION

The amphibian disease chytridiomycosis, caused by the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), has been implicated in declines of amphibian populations and species worldwide (Berger et al. 1998, Longcore et al. 1999, Stuart et al. 2004). Isolating *Bd* from the wild poses some challenges for researchers studying the biology of this infectious pathogen and attempting to develop mitigation approaches. Protocols for isolating *Bd* from amphibians have been published (Longcore et al. 1999, Longcore 2000, Piovita-Scott et al. 2015; see <https://umaine.edu/chytrids/batrachochytrium-dendrobatidis/directions-for-isolation>), and working

from these protocols, we developed an approach that included a unique combination of antibiotics to reduce bacterial contamination.

We systematically assessed the isolation success of our protocol on 2 hylid treefrogs in the genus *Pseudacris*. We also evaluated the relationship between isolation success and *Bd* infection intensity of the frogs, and compared isolation success across tissues collected from 3 anatomical regions where the pathogen is thought to concentrate (Berger et al. 1998, 2005, Puschendorf & Bolanos 2006, Hyatt et al. 2007). Species of these treefrogs are quite common across North America, and some are known to be resistant to chytridiomycosis while carrying high *Bd* infection loads (Reeder et al. 2012). Such frogs may provide

\*Corresponding author: [anthony.waddle@unlv.edu](mailto:anthony.waddle@unlv.edu)

opportunities for isolating and studying *Bd* across a diverse landscape without damaging populations of rare or endangered amphibians.

## MATERIALS AND METHODS

### Media preparations

We used 4 different antibiotics in growth media when isolating *Bd* from frog tissues: kanamycin, ciprofloxacin, streptomycin, and penicillin G. Both kanamycin sulfate and ciprofloxacin were prepared in separate solutions by adding 10 g and 1 g, respectively, to 1 l of autoclaved deionized (DI) water. Streptomycin sulfate (30 g) and penicillin G (20 g) were added simultaneously to 1 l of autoclaved DI water. Aliquots of these solutions were kept frozen before use.

We used TGhL and H-broth media for isolation and growth of *Bd*, following preparation protocols from Longcore (2000); however, a 1% tryptone medium should suffice (Piotrowski et al. 2004). We prepared TGhL in 250 ml quantities to ameliorate issues with media hardening prior to pouring. We added antibiotics to both media types once the temperature of the solutions dropped below 50°C. For each 250 ml of TGhL solution, we added 1.25 ml of kanamycin solution (concentration in media = 50 µg ml<sup>-1</sup>), 2.5 ml ciprofloxacin solution (concentration in media = 10 µg ml<sup>-1</sup>), and 2.5 ml of streptomycin–penicillin G solution (concentrations in media = 300 µg ml<sup>-1</sup> and 200 µg ml<sup>-1</sup>, respectively). For H-broth, we added 10 ml of streptomycin–penicillin G solution (with concentrations in media = 300 µg ml<sup>-1</sup> and 200 µg ml<sup>-1</sup>, respectively).

Each 250 ml solution of TGhL medium made approximately 25 small agar plates (5 cm). We previously experienced desiccation of this medium, so we poured plates (Petri dishes) ca. half-full to maintain appropriate moisture content to facilitate *Bd* growth. Plates were poured under a sterilized laminar flow hood, and once solidified, they were stored in plastic sleeves at 4°C. We also stored H-broth at 4°C.

### Animals

We used 26 *Pseudacris regilla* (potentially *Hyla regilla*, see Duellman et al. 2016) and 32 *P. triseriata*. We collected *P. regilla* from Spring Mountain Ranch State Park, Clark County, Nevada, and acquired *P. triseriata* from Arizona Game and Fish Department

personnel who collected them at Coleman Lake, Coconino County, Arizona. We temporarily housed frogs individually in clear plastic boxes with clasping lids (20 cm width × 36 cm length × 12 cm height) containing ~1 l of aged, dechlorinated tap water, and a small plastic platform for cover and a dry surface. We kept the containers in an environmental chamber set to 19°C under a 12:12 h light:dark photoperiod.

### Determining infection intensity

We determined *Bd* infection intensity of each frog prior to isolation attempts using quantitative real-time polymerase chain reaction (qRT-PCR) of skin swabs as described by Jaeger et al. (2017). Our reactions followed a common assay (Boyle et al. 2004), with infection intensity interpreted as the number of *Bd* zoospore equivalents (ZE) swab<sup>-1</sup>. To quantify infections, we used representative standards of *Bd* isolated from each of the geographic regions from which the frogs were collected (i.e. Spring Mountain Ranch and Coleman Lake) to reduce the possibility that variations in genomic content among *Bd* strains could affect our zoospore counts (Longo et al. 2013).

### Isolation attempts

Prior to isolation attempts, we euthanized frogs following an accepted protocol, wrapped each in a laboratory wipe, and placed them into small resealable plastic bags. We processed all frogs within 24 h and kept euthanized frogs that were not immediately processed at 4°C. We processed frogs on an open, sterilized bench. Forceps, small surgical scissors, and inoculating needles were flame sterilized before each use. For each frog, we used several pairs of TGhL-antibiotic plates and an additional 'master plate.' We divided the master plate into sections labeled thigh, abdomen, and foot. The first plate of each pair (referred to as the cleaning plate) served as a cleaning substrate (see Longcore 2000), while the second plate eventually held the cleaned piece of tissue (referred to as the tissue plate). We placed frogs ventral side up and excised ~4 × 4 mm pieces of tissue (Fig. 1), placing each piece into the appropriate section on the master plate. We subsequently dissected the larger samples into ~1 × 1 mm pieces. These frogs are quite small, so for foot samples we excised the most proximal 1 mm portion of the largest toe of each foot and associated webbing.

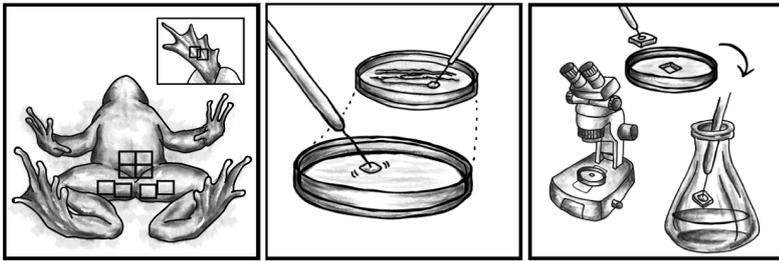


Fig. 1. *Batrachochytrium dendrobatidis* (*Bd*) isolation approach. (a) Tissue is excised in pieces from the abdomen, thigh, and foot (indicated by squares). (b) These tissues are then plunged and dragged through antibiotic agar plates using a sterile inoculating needle and then left on a new antibiotic plate. (c) Final steps of assessing *Bd* presence and growth, followed by transfer to liquid media

We individually transferred pieces of tissue from the master plates to appropriately labeled cleaning plates using an inoculating needle. We plunged (submerged) each tissue into the agar and dragged it across and through the agar about 10 times, with the intent of removing unwanted fungal spores and bacteria (Longcore 2000). We then spread the tissue sample out in a clean tissue plate, which was then wrapped in Parafilm<sup>®</sup>, inverted, and incubated at 23°C. We took care to ensure that plates were fresh enough to provide a small halo of water around the tissue pieces, as observed under magnification; we believed this was especially important because *Bd* is highly susceptible to desiccation (Johnson et al. 2003). For each *P. regilla*, we collected 8 thigh samples, 4 abdomen samples, and 2 foot samples. For *P. triseriata*, we used 4 pieces of tissue from each of these anatomical regions.

We monitored plates weekly over 1 mo for *Bd* growth under 40× magnification, and noted the number of plates in which *Bd* growth was observed. We recorded the number of plates that were contaminated and contaminant type (presumed bacterial or fungal if showing hyphae). Once *Bd* zoospore activity were visible, a chunk of the agar (~1 cm<sup>3</sup>) containing the skin sample with *Bd* was transferred to a 125 ml flask containing 50 ml of H-broth with antibiotics. The identity of *Bd* on plates (as opposed to other fungal species) was confirmed with qRT-PCR.

We determined the role of anatomical region (thigh, abdomen, or foot) on the success of *Bd* isolation for each species independently. We calculated proportions of successful plates for each region per individual and then applied a linear mixed multiple regression model. Frogs were randomly collected from the field, and randomness among samples taken from an individual frog was accounted for via variance-covariance structure. Bonferroni adjustments

were used for multiple comparisons. These statistical analyses were conducted using SAS version 9.4 (SAS Institute). We determined whether *Bd* infection intensities of frogs influenced *Bd* isolation success by assessing Spearman's rho correlations among the proportion of successful plates (those with isolated *Bd* zoospore activity) and the infection intensity (ZE) of the frog from which samples were taken; infection intensity of each frog was divided by its snout-to-vent length (SVL) for size correction.

### Summary of the simplified method

- (1) Remove tissue and dissect into ~1 × 1 mm pieces using surgical scissors and forceps (Fig. 1a).
- (2) Clean tissue by plunging and wiping through agar plate containing 4 antibiotics (Fig. 1b).
- (3) Place tissue on a clean antibiotic agar plate, cover in Parafilm<sup>®</sup>, and incubate at room temperature (~23°C).
- (4) Monitor for zoospore activity under 40× magnification (a compound microscope at up to 100× magnification and sub-stage lighting has also been suggested).
- (5) Transfer to H-broth once zoospore activity are visible (Fig. 1c).
- (6) Refrigerate cultures once clumps of zoospore activity are observed (typically 1 to 2 wk) and transfer to new media within 4 to 6 mo.
- (7) Cryo-archive *Bd* isolate for long-term storage as soon as possible following the protocol of Boyle et al. (2003).

## RESULTS

We isolated *Bd* from 73.0% of *Pseudacris regilla* and 65.6% of *P. triseriata* (68.9% overall). Out of the total frogs used, 77.6% (45/58) tested positive for *Bd* infection ( $\geq 1$  ZE). Of the *Bd*-positive frogs, we isolated *Bd* from 88.8% of *P. regilla* and 70.3% of *P. triseriata* (35/45, 77.8% overall). We also isolated *Bd* from 5 of 13 frogs that did not test positive for *Bd* infection. We found positive trends between *Bd* infection intensity of frogs (ZE/SVL) and the proportion of plates from which *Bd* was isolated for both species (*P. triseriata*,  $\rho = 0.52$ ,  $p = 0.002$ ; *P. regilla*  $\rho = 0.53$ ,  $p = 0.005$ ).

We isolated *Bd* from all 3 body regions (thigh, abdomen, foot) of both species (Fig. 2). Of these body regions, success at isolating *Bd* was significantly higher for foot samples from both *P. regilla* (foot vs. abdomen  $p = 0.010$ ; foot vs. thigh  $p = 0.050$ ) and *P. triseriata* (foot vs. abdomen  $p < 0.000$ ; foot vs. thigh  $p < 0.000$ ). Isolation success did not vary significantly between the abdomen and thigh for either *P. regilla* ( $p = 1.00$ ) or *P. triseriata* ( $p = 0.605$ ).

Contamination from bacteria and unwanted fungi for all plates was low (9.65% of 1554 plates), with remaining plates either containing *Bd* (14.03%) or nothing at all. The most common type of contamination was presumed fungal (76.67% of plates with contamination). We observed contamination on 9.50% of cleaning plates and 8.95% of tissue plates, while 22.4% of the master plates were contaminated. We were able to start *Bd* cultures in H-broth with antibiotics from all plates with isolated zoosporangia.

## DISCUSSION

We successfully used our protocol to isolate *Bd* from populations of *Pseudacris regilla* and *P. triseriata* that had high rates of infection. We isolated *Bd* from each of the 3 anatomical regions from which we extracted skin samples (thigh, abdomen, foot), but our isolation attempts from the feet (toe and associated webbing) were significantly more successful. Webbing from hind feet of ranids has been shown to carry higher *Bd* infections than other body regions and has long been suggested as a primary area to

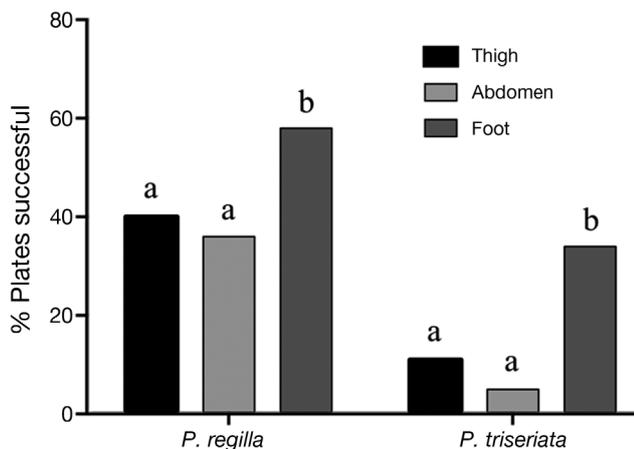


Fig. 2. Percentage of plates from which *Batrachochytrium dendrobatidis* (*Bd*) was successfully isolated, shown by anatomical region (thigh, abdomen, foot) for *Pseudacris regilla* and *P. triseriata*. Letters indicate significant differences within species

sample for *Bd* detection (Longcore et al. 2007). Sampling webbing may prove to be an effective, non-lethal approach for isolating *Bd* (as suggested by J. Piovia-Scott & K. Pope pers. comm.).

*Bd* infection intensity of frogs was positively correlated with our isolation success, indicating that *Bd* isolation may be more successful from more heavily infected individuals. We found that once *Bd* infection intensities reach  $\geq 40$  ZE, we had high levels of isolation success (*Bd* isolated from 74% of *P. triseriata* and 100% of *P. regilla* with such infections). Our success at isolating *Bd* from frogs that tested negative for *Bd* infection was surprising, but was probably due to low intensity infections often being missed using swabbing protocols (Shin et al. 2014). We used an established qRT-PCR method to detect and confirm *Bd* identity, but microscopic examination of freshly collected tissue can also be used (Longcore et al. 1999, Longcore 2000, Longcore et al. 2007). Microscopy may be more efficient than qRT-PCR, particularly if working with only a few frogs, as tissue samples can be excised and viewed immediately (Longcore et al. 2007).

Our early preliminary attempts at isolating *Bd* using common antibiotic combinations (e.g. Longcore et al. 1999, Longcore 2000, Piovia-Scott et al. 2015) were mostly overrun with bacteria, but our combination of 4 antibiotics appears to have been effective at eliminating most bacterial contaminants. We used concentrations of streptomycin and penicillin as described in the literature, and kanamycin and ciprofloxacin as advised by the manufacturer for eliminating bacterial contaminants in cell cultures. Using 5 cm plates in pairs allowed us to isolate and monitor each piece of tissue independently (also see Piovia-Scott et al. 2015), and this alteration from earlier protocols using larger plates and multiple pieces of tissue (e.g. Longcore 2000) may reduce contamination by limiting it to some tissue pieces without affecting others. In a few instances, we observed *Bd* on cleaning and master plates, which highlights the utility of keeping and monitoring all plates for isolations. Growth of *Bd* to the point of easy observation of zoospores and zoosporangia may also be quite slow, and we observed *Bd* presence on many plates only after 3 to 4 wk.

Our adoption and refinement of established *Bd* protocols has benefited our research, and since developing this protocol, we have successfully applied it to isolate *Bd* from White's treefrogs *Litoria caerulea* and relict leopard frogs *Rana onca* used in laboratory experiments, and from American bullfrogs *Rana catesbeiana* captured from the wild.

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