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NOTE

Non-lethal isolation of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) from amphibians

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ABSTRACT: The ability to isolate and purify pathogens is important for the study of infectious disease. A protocol for isolating *Batrachochytrium dendrobatidis* (*Bd*), a lethal pathogen of amphibians, has been available for over a decade, but the method relies on sacrificing infected animals. We validated a non-lethal protocol for Bd isolation that uses biopsy punches from toe webbing to collect skin samples from live amphibians in remote field locations. We successfully isolated *Bd* from the Cascades frog *Rana cascadae* and found a positive association between *Bd* infection and probability of *Bd* growth in culture. Recapture rates of sampled animals suggest that our isolation protocol did not affect frog survival. The ability to collect isolates from live animals will facilitate investigations of the biology of *Bd* and enhance amphibian conservation efforts.

KEY WORDS: Amphibian declines · *Batrachochytrium dendrobatidis* · Chytridiomycosis · Pathogen isolation · Biopsy

INTRODUCTION

Pathogen isolation is central to the study of infectious disease. Since the 18th century, when Louis Pasteur and Robert Koch advanced the field of microbiology, developing techniques to isolate, identify, and characterize pathogens has been paramount in establishing multiple biomedical and scientific fields (Brock 1988, Ullmann 2007). Today, isolation techniques remain critical because a wide variety of investigations require purified pathogen isolates, including studies focused on understanding disease emergence, distribution, spread, and pathogenesis (Voyles et al. 2009). In addition to facilitating investigations of the basic biology of disease, purified pathogen isolates can help inform risk assessments, identify susceptible hosts, and determine the best intervention strategies (Voyles et al. 2009, Langwig et al. 2015).

The availability of purified pathogen isolates has played a critical role in advancing research on chytridiomycosis, a lethal disease of amphibians. The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is one of the causative agents of chytridiomycosis (Berger et al. 1998, Longcore et al. 1999), and has contributed to declines and extirpations of hundreds of amphibian species worldwide (Skerratt et al. 2007, Catenazzi 2015). It has therefore been the subject of intense investigations on phenotypic characteristics, virulence traits, phylogenetics, and genomics (Farrer et al. 2011, Voyles 2011, Rosenblum et al. 2013, Piovia-Scott et al. 2015, Byrne et al. 2016). The resequencing of *Bd* isolates collected across the globe has resulted in the advancement of our understanding of

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the evolutionary history and emergence of *Bd* (Rosenblum et al. 2013). Such investigations have relied on access to a library of *Bd* isolates (Voyles et al. 2009, Farrer et al. 2011, Rosenblum et al. 2013, Byrne et al. 2016). Continued collection of isolates over time may provide valuable insight on the evolution of virulence as host populations become extinct or transition into an enzootic state (Voyles et al. 2018).

Longcore et al. (1999) were the first to develop a technique to isolate *Bd* from an infected dendrobatid frog, which has since been used by many researchers for obtaining new *Bd* isolates. The protocol involves excising pieces of infected skin from recently-deceased or sacrificed animals, cleaning the tissue, and plating the samples on nutrient-rich antibiotic agar where *Bd* can grow before it is transferred into liquid media (Longcore 2000). However, this protocol has an important shortcoming for the collection of new isolates from wild populations in that it requires sampling from a deceased host. While this can be reasonably achieved for some populations through encountering a moribund or recently-deceased amphibian host, or sacrificing individuals from populations known to be infected with *Bd*, obtaining *Bd* isolates from lethal sampling can present ethical dilemmas in small or declining populations. Because of this, researchers have pursued non-lethal means for isolating *Bd* (e.g. Fisher et al. 2018). In this paper, we evaluate a non-lethal protocol that we developed for isolating *Bd*, focusing on predictors of successful isolation and survivorship of animals used in the isolation protocol in field conditions.

In infected amphibians, the zoospores colonize the superficial layers of the epidermis, especially the stratum corneum (Longcore et al. 1999, Berger et al. 2005). *Bd* is known to disproportionately colonize the skin of the abdomen, pelvic patch, and feet (Berger et al. 2005, Puschendorf & Bolanos 2006, Hyatt et al. 2007, Waddle et al. 2018). Thus, collecting *Bd* isolates from toes or the webbing between toes could be an effective way to obtain *Bd* isolates from live amphibians, and thereby alleviate the need to sacrifice individual hosts. Therefore, we examined the efficacy of isolating *Bd* from biopsy punches from toe webbing. In addition to validating a non-lethal isolation protocol, we compared the rates of successful isolation across amphibian life stages, with different sizes of tissue samples, and among multiple sites for Cascades frogs *Rana cascadae*. Finally, we used recapture rates of marked animals to evaluate the effect of sample collection on survival.

MATERIALS AND METHODS

Study species and sites

We focused on populations of Cascades frogs infected with *Bd*. We selected this species for multiple reasons. First, *Rana cascadae* provides a compelling example of a susceptible species that experienced disease-induced declines when *Bd* emerged in northern California, USA (Piovia-Scott et al. 2015, de León et al. 2017). Second, although declines have occurred, we were able to target relatively large populations such that we could avoid significant population-level effects if tissue collection reduced survival. Third, we used sites where long-term monitoring of population dynamics and *Bd* infection patterns could help us sample when the likelihood of encountering *Bd* was high. Fourth, we could test this protocol in conjunction with an ongoing population monitoring effort.

Field surveys and sample collection

In 2015–2016, we visited 2 Cascades frog populations. We collected samples from frogs captured at Found Lake and Section Line Lake in the Trinity Alps Wilderness in Northern California, which have been *Bd*-positive since at least 2008 (Piovia-Scott et al. 2011). We captured frogs by hand, using a fresh pair of gloves, an inverted plastic bag, or by using a clean net (Phillott et al. 2010). We recorded the sex, mass, and snout-to-urostyle length (SUL) of captured frogs, and we noted any clinical signs of infection (Voyles et al. 2009). In addition, we collected a diagnostic sample for each captured animal by swabbing the feet, thighs, and abdominal region with a sterile cotton-tipped swab (Piovia-Scott et al. 2011, 2015, Perez et al. 2014). We transported the *Bd* diagnostic samples to the laboratory for analysis with quantitative polymerase chain reaction (qPCR; described below).

Tissue collection in the field

We focused on collecting skin tissue samples from the webbing found between the toes of Cascades frogs. For 40 individual frogs from 2 lakes (20 frogs lake^{-1}), we collected 1 skin sample from each frog. We used sterile biopsy punches (Integra Miltex) to collect either 1.5 mm (juveniles and adults) or 3.0 mm (adults only) diameter skin tissue samples. We mani-

pulated the skin samples using an inoculating needle that was flame-sterilized between uses. We first 'cleaned' the sample by submerging and repeatedly drawing it through sterile TGhL agar (1000 ml H_2O, 16 g tryptone, 2 g gelatin hydrolysate, 4 g lactose, 10 g agar) with antibiotics (300 mg l^{-1} penicillin-streptomycin) to prevent growth of bacteria, yeast, and other contaminants (Longcore 2000). We then placed the intact clean tissue sections onto the surface of 90 mm TGhL agar plates with antibiotics. Up to 4 tissue samples were stored on 1 plate. We stored the samples in a cooler with ice packs for transport to the laboratory.

Isolate growth in the laboratory

We followed standard culturing procedures (Longcore 2000) which include starting cultures on TGhL agar plates with antibiotics and transferring contamination-free *Bd* growth into TGhL broth. We incubated plates at 21–22°C and monitored the samples using daily inspection with an inverted microscope (Nikon Eclipse TS100), looking for zoospore activity, *Bd* colony formation, and fungal or bacterial contamination (Piotrowski et al. 2004). If we observed contamination, we removed the contaminated area from agar plates as soon as it was detected (Longcore 2000). Once we observed either motile zoospores or a *Bd* colony without contamination, we transferred a subsample of the culture to fresh TGhL liquid media without antibiotics. Hereafter we will refer to observed motile zoospores as '*Bd* growth' and *Bd* successfully grown in pure culture without antibiotics as 'isolation.'

qPCR analysis of *Bd* infection intensity

We extracted and stabilized DNA from swabs (Boyle et al. 2004, Hyatt et al. 2007) using PrepMan Ultra (Life Technologies) which we diluted 1:10 with molecular grade H_2O. We added 2.5 µl of the DNA to each well with 10 µl of reagent for a total reaction volume of 12.5 µl. DNA samples were analyzed in triplicate with a CFX real-time PCR system (Bio-Rad Laboratories). Negative controls and standards of known concentration were included in each plate. To quantify pathogen load, we used oligonucleotide standards (gBlocks, Integrated DNA Technologies) to estimate the number of internal transcribed spacer (ITS) copies on each swab. These copy numbers were converted to zoospore equivalents (ZE) using calibrations developed by running zoospore standards and oligonucleotide standards on the same qPCR plate (J. Piovia-Scott unpubl. data). To stabilize the distribution of ZE, we log-transformed ZE prior to analysis, with a constant added to avoid taking the log of 0 (the constant was the smallest non-0 ZE value divided by 2). Infection intensity data from 1 of the animals used for *Bd* isolation are lacking due to a missing or mislabeled swab sample.

Capture–mark–recapture and survival estimates

As part of ongoing studies, we marked Cascades frogs using passive integrated transponder (PIT) tags for larger individuals (≥32 mm SUL). We returned to the sites to search for marked individuals 1 yr after we collected our biopsy samples. To obtain survival estimates, we systematically searched the lake shoreline and checked all captured frogs for PIT tags (Pope 2008) and visual evidence of biopsy punch sampling. We were not able to PIT tag juvenile frogs, and therefore they are not included in the recapture analyses.

Statistical analyses

We conducted all analyses using R statistical software (Version 3.4.3; R Core Team 2016). We used binomial generalized linear models to analyze the effect of life stage, site, and infection intensity on both *Bd* growth and isolation success. Because life stage was confounded with punch size (9 adult frogs and no juveniles were sampled using the larger 3 mm punch), we excluded the animals sampled with a 3 mm punch from this analysis, giving a sample size of 31 animals. We used a separate set of analyses to evaluate the effect of punch size on *Bd* growth and isolation success. We used only adult animals (n = 15) for these latter analyses because juveniles were not large enough to be sampled with the larger 3 mm punch; size and infection intensity were used as covariates in these analyses.

The availability of PIT-tagged individuals allowed us to compare survival rates for tissue-sampled (n = 15) and control (i.e. unsampled, n = 116) frogs. We used the binomial distribution to generate 95% confidence intervals of recapture rates to compare survival in the 2 groups, and conducted a Fisher's exact test to determine if there were statistically significant differences in recapture rate.

RESULTS

We successfully isolated *Bd* from 14 Cascades frogs using biopsy punches on toe webbing (Table 1). Although we observed *Bd* growth via microscopy in 87.5% (35/40) of the samples that we collected, we were not able to obtain pure *Bd* isolates from 21 of these samples because other species of fungi contaminated the skin samples.

We found that 79% (31/39) of the frogs tested positive for *Bd* with a mean infection intensity of 356 ZE (SE = 74). There was a positive association between infection intensity and probability of *Bd* growth (χ^2 = 4.2, df = 1, p = 0.04). However, there was no significant association between infection intensity and probability of *Bd* isolation (χ^2 = 0.66, df = 1, p = 0.42).

We also tested for differences across amphibian life stages, biopsy punch sizes, and between sites. We found that 1.5 mm samples collected from juvenile frogs were more likely to show *Bd* growth (juveniles: 18/25; adults: 1/6; χ^2 = 11.0, df = 1, p = 0.001), and produce purified *Bd* isolates (Table 1; χ^2 = 4.3, df = 1, p = 0.04) than the same size samples collected from adults. We found that the 3.0 mm biopsy punch size had a greater probability of *Bd* growth than the 1.5 mm (1.5 mm: 1/6; 3.0 mm: 6/9; χ^2 = 5.3, df = 1, p = 0.02) and increased isolation success (Table 1; χ^2 = 8.0, df = 1, p = 0.005) in samples collected from adult frogs. Lastly, we found a difference between the 2 Cascades frog sites in the probability of *Bd* growth (Section Line Lake: 13/18; Found Lake: 6/13; χ^2 = 9.17, df = 1, p = 0.002), but not isolation success (χ^2 = 0.50, df = 1, p = 0.48).

Table 1. Proportion of successful isolation (*Batrachochytrium dendrobatidis* isolated into pure culture) by site, punch size, and life stage (successful isolation/total sampled). Juvenile Cascades frogs were only sampled with the 1.5 mm biopsy punch

Site	Juvenile	Adult 1.5 mm	Adult 3.0 mm
Found Lake	4/12	0/1	3/7
Section Line Lake	5/13	0/5	2/2
Total	9/25	0/6	5/9

Table 2. Recapture rates and 95% CI for survival of Cascades frogs, estimated using the binomial distribution

	Initial captures	Recaptures	Rate of recapture	95% CI (lower, upper)	SE
Biopsy sampled	15	6	0.40	0.16, 0.68	0.1264
Not sampled	116	40	0.34	0.26, 0.44	0.044

We recaptured Cascades frogs at relatively high rates (Table 2). We recaptured 40% of the sampled (i.e. biopsied) frogs and 34% of the unsampled frogs. Confidence intervals for these 2 groups overlapped broadly, suggesting no difference in survival; the results of a Fisher's exact test did not indicate significant differences in recapture rate (p = 0.77).

DISCUSSION

We validated a non-lethal method to maximize the likelihood of isolation of *Bd* from live animals in the field (for the protocol, see the Supplement at www.int-res.com/articles/suppl/d129p159_supp.pdf) and successfully isolated *Bd* from frogs in remote field locations. Not surprisingly, *Bd* infection intensity influenced the probability of observing *Bd* growth. Therefore, this protocol will be most useful for researchers when the *Bd* infection status of target populations is known. In addition, life stage and the size of the biopsy punch also influenced the probability of obtaining an isolate. Given that multiple species of juvenile frogs are frequently more heavily infected than adults (Russell et al. 2010, Vredenburg et al. 2010, Piovia-Scott et al. 2011), sampling during this life stage may also improve the chances for successful isolation.

One of the main factors that limited our ability to purify isolates was contamination by other fungi. In some cases, we were able to cut out contamination from agar plates and grow *Bd* in pure culture. Unfortunately, in many cases the contamination grew too quickly, leaving us unable to isolate *Bd*. It is likely that cleaning the tissue in the field is not as effective as doing so in the lab because of ambient microbes and other unsterile conditions. We recommend careful cleaning of the tissue prior to plating, shielding as much of the process as possible from ambient microbes using a large plastic bag, and taking multiple tissue samples from a target population to increase the likelihood of obtaining at least 1 uncontaminated isolate. We chose to present *Bd* growth as a result alongside isolation because *Bd* isolation is influenced by other factors, such as sterile technique, that may not be related to the abundance of *Bd* in the sampled tissue; all cases of successful isolation exhibited *Bd* growth.

This sampling protocol does not appear to adversely affect recapture rates or survival of sampled individuals. We did not observe any signs of distress or

mortality in animals subjected to tissue sampling. Moreover, we found no visible evidence of the biopsy punch in sampled animals captured a year after tissue collection. This suggests that there were no differences in survival as a result of collecting skin samples. Thus, we propose that collecting a biopsy punch is reasonably safe and does not appear to substantially increase the risk of mortality in *Bd*-infected amphibians. In order to minimize damage potentially resulting in mortality, we chose to only take 1 tissue sample per individual. Since established methods (Longcore 2000) use deceased or sacrificed animals, multiple tissue samples can be taken from 1 individual, therefore increasing the likelihood of obtaining a pure culture from any single animal. The method we describe prioritizes isolation success at the spatiotemporal level instead of the level of the individual animal. The risks and benefits of lethal vs. non-lethal methods should be considered on a case-by-case basis.

Understanding *Bd* dynamics on fine spatial scales remains a priority for researchers (Voyles et al. 2009, Byrne et al. 2016). To date, multiple questions regarding disease dynamics within a single host species, or within individual sites and populations, are understudied, due (at least in part) to the challenges associated with isolate collection. Recently, new methods for genotypic profiling of *Bd* from swabs were optimized and will greatly advance investigations on the phylogenetics of *Bd* (Byrne et al. 2016). However, molecular analyses and genotypic profiling may not fully capture pathogen functional and phenotypic characteristics important to disease development. Integrating functional and phenotypic characteristics of multiple *Bd* isolates will complement cutting-edge genomic research in understanding *Bd* virulence and disease development.

We found that non-lethal isolation of *Bd* from infected animals can be performed in the field, using a small sample of skin tissue, and without serious impacts on survival. This approach provides a wide range of opportunities for *Bd* isolate collection. Therefore, this method will help obtain a more complete understanding of *Bd* dynamics without compromising already distressed host populations or vulnerable species. As *Bd* and other fungal pathogens (e.g. *Batrachochytrium salamandrivorans*) continue to threaten amphibians, continued efforts to isolate and characterize these pathogens can be used to enhance monitoring and move conservation efforts forward.

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