FIELD-SAMPLING PROTOCOL FOR *BATRACHOCHYTRIUM DENDROBATIDIS* FROM LIVING AMPHIBIANS, USING ALCOHOL PRESERVED SWABS

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INTRODUCTION

Chytridiomycosis, the infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), is responsible for population declines and extinctions of amphibians in many parts of the world. *Bd* is an aquatic fungus that affects the keratinized stratum corneum of adult amphibians and the keratinized or pre-keratinized mouthpart tissues of anuran larvae. No information is available concerning the pathology of *Bd* in larval salamanders. The mechanism by which chytridiomycosis results in the mortality of amphibians is unknown but osmoregulatory inhibition and alterations of blood solute concentrations are suspected. Unlike most other aquatic fungi, *Bd* is not known to produce a resting stage, but *Bd* zoospores (the motile infectious stage) remain detectable, and can presumably remain viable, in water for up to six weeks. Complete drying of media and temperatures above 30° are lethal to *Bd*, but it can persist in amphibians at cold temperatures (4–8°) although proliferation of *Bd* at these low temperatures is greatly reduced. Optimum conditions for *Bd* proliferation in controlled laboratory settings appear to be 22°, neutral pH, and 100% moisture saturation.

In order to understand the global and local distribution of the fungus, information on its presence or absence at sites and among species around the world is critical. The purpose of this publication is to inform researchers and professionals of a non-lethal methodology for collecting superficial samples from the skin of wild amphibians. These samples are then used to test for the presence of *Bd* using a diagnostic PCR assay, a molecular technique to detect the infection status of an individual. "PCR" stands for Polymerase-Chain Reaction; this reaction is a critical part of the molecular technique to test for the presence of DNA from *Bd*. The PCR assay is currently the best method available for detection of *Bd* in wild populations due to its sensitivity and the ability to detect even very light infections. The general outline of this procedure is to use a cotton swab to take a superficial sample from the skin of an amphibian, preserve the sample in ethanol, and send it to an established molecular laboratory for the diagnostic assay. The primary benefits of this PCR assay for the detection of *Bd* are its extreme sensitivity and the ability to rapidly and non-lethally collect and analyze samples from numerous amphibians in the field.

This document will instruct researchers and professionals on how to sample a living frog for *Bd* and preserve the sample for subsequent shipment to a molecular laboratory. We will discuss how to conduct sampling in order to minimize the possibility of transmission of the pathogen among individual amphibians and amphibian communities, and to avoid contamination among samples. Avoiding contamination across samples is crucial, since it can lead to diagnosis of the presence of *Bd* in samples from which it is absent, producing incorrect and misleading results (false positives).
A false-positive result occurs when the animal itself is *Bd* negative but the assay returns an erroneously positive result, probably due to contamination. The PCR assay itself is unlikely to produce a false-

positive result, however, because the PCR assay is extremely sensitive and can detect as little as one zoospore, contamination among samples can lead to false-positive results. False-negative results occur when the animal is actually positive but the assay result is negative. False-negative results can be reduced by using the PCR assay with carefully collected samples. They can still arise, however; for example, it is possible to sample an amphibian with a light infection, with few fungal pathogens in its skin, and miss the infected areas. Sampling multiple areas of the body that are common sites of infection, such as the hands, feet, thighs and venter, can minimize false-negatives.

The swabbing method apparently does not injure the amphibian, as only flushing of the toe-tips has been observed, but the effects of capture and subsequent swabbing have not been definitively tested. If the animal is to be collected as a museum specimen, it should be sampled for *Bd* prior to preservation. Similarly, any frogs found dead or dying in the field should be sampled by swabbing. All dead amphibians encountered should be preserved as museum specimens as soon as possible after a sample for a *Bd* assay is taken. Preserved specimens can also be examined using other methods of detection for *Bd,* such as light microscopy or histological examination, although these methods are less sensitive than the PCR assay.

METHODS

Note: The protocol described below uses ethanol as a DNA-preservative, and suggests using swabs with a wooden shaft. Some laboratories (e.g., those using Real-Time PCR technology) prefer simply place swabs directly into the sample tube, with no preservative. For samples that will be stored for long periods of time (e.g., weeks) before analysis, or for samples that will be exposed to heat and/or high levels of humidity (e.g., tropical field work), we recommend that ethanol be used to preserve any possible DNA in the sample. For Real-Time PCR we recommend use of swabs with a plastic shaft.

Swab Collection Procedure

Swabbing is best conducted by two people and requires the following materials:
- 70% EtOH
- cotton tipped applicators ("swabs")
- vials (~2ml w/ self-sealing screw caps; Nunc® Brand microcentrifuge tubes are recommended)
- powder-free latex or nitrile gloves
- alcohol sanitizer for hands (Purell brand is recommended)
- water- and alcohol-proof pens (VWR brand is recommended)
- Waterproof notebook (Rite in the Rain® brand is recommended)
- sharp-pointed dissecting probe
- waste bag or container
- collection bags
- closeable bags (Zip-Loc® or Whirl-Pac® style recommended)
- vial storage boxes
- Bleach solution: 1 part commercial bleach (5% sodium hypochlorite) to 9 parts water

All critical equipment can be purchased through any biological supply company such as Fisher Scientific, Ben Meadows, Forestry Suppliers Inc., Ward's Natural Science (vials, vial containers, swabs, Rite-in Rain, Whirl-Pac bags, gloves). All other supplies can be purchased at most retail stores.

Person 1 will handle the amphibians, this person should carry only:
- gloves (1 pair per animal captured, 1 box for approx. 50 captures)
- waste bag/container

Person 1 is to capture and handle the amphibian, collect the fungal sample, and take any measurements desired from the animal (i.e., mass, length). It is very important that gloves be changed between every animal to prevent contamination across samples and to reduce the possibility of transmitting *Bd* among amphibians handled. Gloves must be put on and removed with care to prevent unwanted dispersal of this pathogen to clean equipment or habitats.

Person 2 is responsible for taking data, labeling vials, and carrying equipment. This person will carry:
- swabs
- vials (pre-filled with ~1mL EtOH)
- alcohol sanitizer for hands
- pens and pencils
- dissecting probe
- collection bags
- waterproof notebook

Person 2 is responsible for keeping sterile items, such as vials and swabs, protected from contamination, and recording all data. Person 2 should still wear gloves in case of accidental contamination of hands by spilling the sample vial or accidental contact with an amphibian.

Procedure:

Preparation:
1. Equipment check, a thorough checklist is advised
2. Organize equipment in field vest(s) for easy access
3. Prepare data pages to include data to collect
4. Begin survey
5. Person 1 puts on clean gloves to capture amphibian
6. Person 2 put on clean gloves in case of accidental contamination of hands, and readies all equipment needed (see list)
7. Walk transect to visually encounter amphibians

Capture:
1. Only Person 1 should attempt to capture amphibian
2. Attempt to capture amphibian as efficiently as possible
3. Minimize handling time for the amphibian, and minimize any contact you may have with any substrate. A clean frog will result in a better sample. However, if the animal is dirty after capture do not wash it. This is suggested for three reasons:
    a) rinsing free zoospores off skin may reduce chances of detecting light infections.
    b) Washing off the animal may introduce large amounts of pathogen into the aquatic environment and facilitate transmission.

c) washing the animal in potentially contaminated water might introduce zoospores onto its surface, resulting in a false positive.

Person 2 can use this time to record capture data/field notes, and label collection vial (species, date, location, number relating to field notes), or ready equipment for swabbing. A sharp dissecting probe is used to label the vial when heavy rain prohibits the use of markers. As a precautionary measure, since many markers are soluble in alcohol, all data written on vials are permanently etched into vials with a dissecting probe upon return from the field.

Person 1 will position the amphibian for swabbing. In the case of frogs, grip the animal by both legs anterior to the hind legs, with the ventral side up to allow access to the ventral side, feet and hands. These areas commonly have a high concentration of *Bd* sporangia in infected animals and will maximize the sensitivity of the assay. Position and grip on a small individual may need to be altered to swab feet first, then pelvic patch and hands.

Swabbing:

1. To swab an anuran, Person 2 first hands Person 1 a clean swab. Person 1 swabs each: rear foot, ventral surface of thigh, and ventral abdominal surface, in that order, using a sweeping motion along the length of each of the five areas to be swabbed. Five sweeps of each area, for a total of 25 sweeps per individual, will be sufficient to sample the animal.
2. Salamanders must be sampled differently because positioning them firmly in the hands is not possible. Three methods have been used with success, and the method used will vary according to the behavior of the species:
    a) If the salamander cooperates, the swab can be placed under the ventral surface, behind the front legs; and then dragged under the animal. This process is repeated up to ten times.

    b) The salamander can simply be picked up with the swab and allowed to hang on while you turn the swab. More contact and longer contact time with the swab should increase the probability of detection, but no relationship has been established.

    c) Procedures Aand C work well for Bolitoglossine and other arboreal salamanders. For salamanders that will not cooperate with the first two sampling methods, place the salamander into a clean collection bag, let salamander move about in the bag, and subsequently swab the inside of the bag. This technique is best for fossorial salamanders such as ambystomatids and *Oedipina* spp.

Preservation of the sample:

1. Person 2 holds an open, alcohol filled, pre-labeled vial as Person 1 carefully inserts the used swab into the vial so that the cotton tip is suspended just above the bottom of the vial.

2. Person 1 then carefully breaks the end of the applicator off into the vial (wooden shaft swabs only; for plastic shafts use scissors to cut shaft appropriately).

3. Finally, Person 2 secures the cap to the vial, assures that all information has been recorded, and stores the sample in a closeable bag labeled with the date, time, and location.

Disposal of contaminated equipment:

1. Dispose of the remaining portion of the swab into waste bag/container
2. Carefully remove gloves by pinching around the opening, and remove glove by inverting it as you remove it. You now have both contaminated gloves in one hand. With your clean ungloved hand, once again pinch the ring around the opening of the glove remaining on your hand, and remove glove by inverting it as you remove it.
3. Dispose of gloves in waste bag/container. By removing your gloves in this fashion, pathogens are trapped inside the inverted gloves and are less likely to be transmitted to other samples, other amphibians, equipment, or into the environment.

Repeat this methodology, using fresh gloves, for all amphibians to be sampled.

If accidental contact with contaminated equipment occurs:

1. Dispose of any equipment that can be easily replaced, such as bags or swabs, by placing it in the waste bag/container.
2. Use alcohol or flame to sanitize other gear such as calipers or scissors. Use alcohol sanitizer for hands and sensitive equipment (scales, calipers, scissors).

Keep in mind that even a tiny trace of DNA can be detected by the diagnostic PCR assay, and that DNA will *not* be eliminated by alcohol. Preventing cross-contamination between samples is crucially important.

At the end of the survey all gear and materials in the waste bag must be decontaminated. This is especially important before disposing of the waste bag, or before traveling between survey sites. Use a bleach solution (1:9 bleach:water) for decontamination of equipment, but be careful not to get any bleach on vials, gloves or swabs. Bleach will quickly degrade the DNA in a sample, and invariably produce a negative result, even if the animal swabbed is positive.

Before driving to other locations, vehicles should be washed thoroughly, paying special attention to remove mud on tires and undercarriage.

Considerations:

1. New gloves must be worn for each capture. It is not acceptable to simply sterilize hands with alcohol. Using alcohol will kill the pathogen and prohibit transmitting disease to other amphibians, but it will also preserve the pathogen's DNA. Preserved pathogen DNA on the hands could contaminate subsequent samples, thus producing potentially false-positive results.
2. The animal should be swabbed before any measurements such as mass are taken. Such measurements should be taken while the animal is in a clean collection bag, so that equipment is not contaminated and infectious particles are isolated in the bag.

EXAMINATION OF CARCASSES BY LIGHT MICROSCOPY (IN VIVO)

Materials:

- Skin scrape from preserved animal
- Compound light microscope w/camera
- Slides and coverslips
- Deionized water and dropper

- 70% EtOH
- lens wipes
- immersion oil (optional for 100x magnification)

Considerations:

1. The only way to get a false-positive result with this procedure is by accidentally examining tissue from a different animal. Care must be taken to properly label all specimens and samples. Specimens should be rinsed to remove all foreign tissue from its surface.
2. False-negative or false-positive results can arise due to the misdiagnosis of *Bd* in amphibian skin, thus, this procedure should only be done by a skilled professional.
3. Samples should be permanently documented. This may be done by preparing a permanent slide mount of the unstained skin or taking photomicrographs of infected tissues to document infection.

Procedure:

1. Prepare a clean slide with 2 drops of deionized water, and have a clean coverslip ready.
2. With a sharp razor blade, scrape off a small section of the stratum corneum from the feet, hands, and/or ventral patch. Ideally, only the stratum corneum is removed, but often, some of the underlying stratum granulosum remains attached. The stratum corneum readily sloughs off in large sections in infected animals, but may be hard to remove in healthy amphibians.
3. Position the removed tissue in the water on the prepared slide so the stratum corneum faces the objective lens of the microscope, and place coverslip over the tissue and water.
4. Blot the edges of the coverslip with a lens wipe. This serves to remove excess water and flatten the skin between the slide and coverslip. This will make it easier to examine and photograph the sample.
5. Examine the tissue under the 40x objective lens for the presence of thalli and sporangia of *Bd.*
6. To document photographically, locate the most obvious examples of *Bd* thalli and/or sporangia on the sample. Photographs, or digital files, should be labeled with the catalogue number and species of the specimen, and submitted along with the specimen to a museum collection.

SAMPLING DESIGN

Because sampling for *Bd* in wild populations can be time consuming, and because PCR assays are expensive, there are some considerations related to sampling that should be weighed by the investigator. The goals of the researcher will determine how the samples are collected and analyzed. Three common goals when sampling amphibians for the presence of *Bd* are: 1) To detect presence of *Bd* at a site, 2) To detect presence of *Bd* in a particular species, or among species and individuals at a site or 3) to determine the frequency of infected individuals (prevalence) at a site(s).

Sampling considerations to maximize value of data for a given goal:

1. For surveys to document presence of *Bd* at a site:
2. Collect individual swab samples, as described above, then instruct the laboratory to combine them for analysis. This procedure can greatly reduce the expense of testing. Do not compromise this procedure by placing more than one swab sample in a nunc tube, and do not ever swab more than one animal with a single swab.
3. For surveys to document presence of *Bd* in a particular species or group of amphibians at a site:
4. Place individual sealed nunc tubes with swab samples from up to 10 individuals into a single bag, to be sent to the diagnostic laboratory where the lab can combine portions of each frog sample into one

sample for analysis. This will test for presence of *Bd* in the group of 10 amphibians for the cost of a single assay. These should be grouped according to species, location, and time. This will reduce the number of PCR assays that must be conducted, and thus reduce time and cost of the results. It is important that the lab do the combining to prevent contamination so that these samples can be analyzed separately (if necessary) in the future.

5. If some or all batches are *Bd* positive it means that at least one amphibian from each positive batch is *Bd* positive, but the infection status of each individual in the batch is unknown.
6. If assay results from individuals within batches are now of interest, re-assay individual swabs from positive batches only. (Note: this will require two samples, one to assay with a group of swabs (batch) and one to assay individually. Acquiring two samples from one individual can be accomplished by: 1) splitting samples before conducting the assay 2) taking duplicate samples in the field or 3) amplifying DNA from each sample before splitting.
7. Carefully consider the scope of the research before combining samples for analysis. Valuable data regarding prevalence of infection among individuals, groups or habitats will be lost after samples are combined if a duplicate sample was not procured. On the other hand, processing an inadequate number of samples when it is suspected that *Bd* may be absent will result in useless data.

<u>For surveys to document prevalence of infection among species or individuals:</u>

1. Collect samples for individual amphibians, as described earlier.
2. Send all samples to lab for individual testing.

Considerations:

1. In order to increase the likelihood of detection of *Bd,* and to improve the statistical power of the study, all sampling designs will benefit from large sample sizes. Small sample sizes may result in false-negative results for a site or species.
2. To avoid wasting time and money, and potentially losing valuable data, the investigator must carefully consider the scope of the survey before sampling begins, and before any samples are combined.
3. Remember that the PCR assay is very accurate, so a false-positive result is unlikely if no contamination has occurred in the field.
4. False-negative results are always a possibility because of simple sampling errors that may occur while sampling a single amphibian, or sampling from a population of amphibians

Considerations for adequate sampling:

The simplest statistical model for infection by the chytrid is that it is a binomial process: Each individual has a completely independent probability of being infected by the organism, with P(infection) = the prevalence of the organism in the population (number of infected animals/total number of animals). This is almost certainly not completely correct for most species, as it is known that the probability of infection depends on things such as individual behavior, with individuals sharing retreat sites likely to transmit the infection, and thus influence each others' probability of being infected. However, it is probably adequate as a first approximation, and for simple hypothesis testing.

It is well known that species vary in their susceptibility to chytridiomycosis, and that the prevalence of infection by *Bd* can vary among species when it is endemic. Because of this, it is not very reasonable to combine samples taken across multiple species and perform simple statistical tests or estimates of prevalence on them, since the outcome may depend more on the composition of the sample than on the abundance of *Bd* in the habitat being surveyed. This applies whether or not the vulnerability of the species

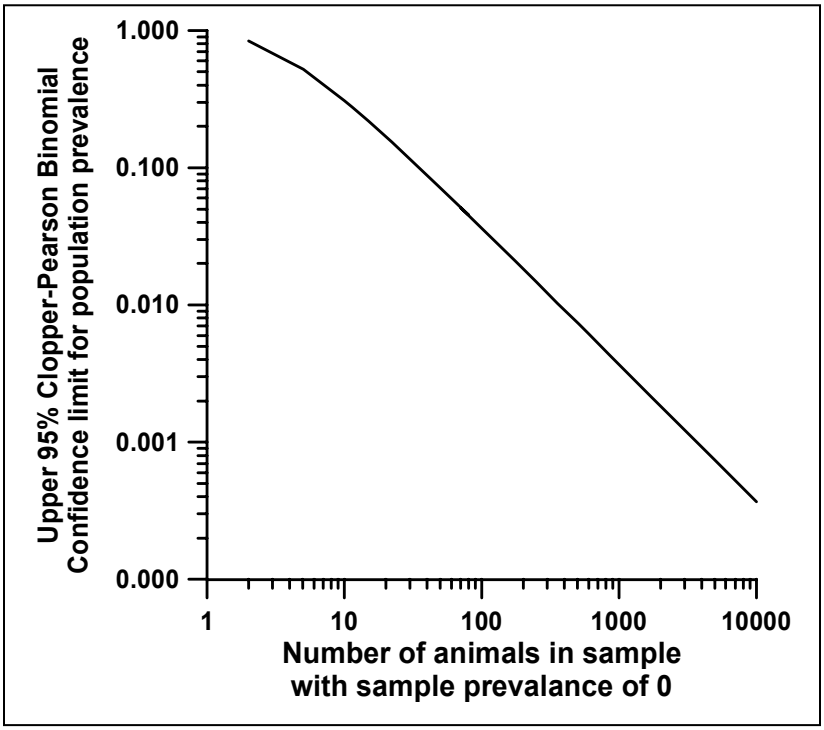
being surveyed to infection by *Bd*, or their susceptibility to chytridiomycosis, is known. All evidence to date suggests that these will vary among species, so that any combined estimate of prevalence will underestimate it for the more vulnerable species and overestimate it for less vulnerable species. This is a particular problem when *Bd* has not been detected in an area; including species that are innately immune to *Bd* infection in aggregated samples could lead to greatly overinflated estimates of statistical power to detect *Bd* if it were present.

The estimated prevalence of *Bd* in a population is simply the number of animals shown to be positive divided by the total population size. The variance of a binomial distribution is *p(1-p)*, so a normal approximation to the 95% confidence limit for *p* can be given as:

$$p \pm 1.96\sqrt{\frac{p(1-p)}{n}}$$

where n is the number of samples taken to estimate *p*.

The usual rule of thumb is that the normal approximation should only be used when np>10 and np(1-p)>10. This always rules out using it for the special case where *p* is estimated to be zero, i.e. there are no positive individuals detected in the sample. For this case, various computer programs can be used to calculate the correct 95% binomial confidence limits for the data, or consult the following figure:



This illustrates the very low power that small samples have to demonstrate that Bd is absent from any population. For example, testing 10 animals, assuming that the effectiveness of the testing procedure at detecting infections is 100%, and finding none infected, will give a 95% confidence interval for true prevalence of *Bd* in the population of from 0 to approximately 0.3 or 30%. It is never possible to prove that *Bd* is absent from a population, but perhaps demonstrating that the upper 95% confidence limit for its prevalence is less than 1% is adequate. Reading the above graph, we can see that this requires a sample size of over 300 (in fact, 366) individuals. Ideally, these should all be of the same species, for the reasons

already discussed, and the results will apply only to that species. If a sample contains a mixture of species, particularly when the innate resistance of the species that make it up to *Bd* is unknown, the true 95% confidence interval cannot be estimated but its upper bound will always be greater than that shown above, because some of the species will be more innately resistant to *Bd* than will others, and some may even be innately immune, so that their presence in the sample adds no information, but only inflates the sample size.

Choosing a more realistic goal, a sample of 35 uninfected individuals of a single species is adequate to demonstrate that the prevalence of *Bd* in that population of that species is probably below 10%, and a sample of 72 is enough to show that it is probably below 5%. When sampling for the first time in a fauna from which *Bd* may be absent, it is important to select species that are known (because they are known to be susceptible to *Bd* in other parts of their range) or suspected because of taxonomic, ecological, and behavioral similarity to susceptible species, to be susceptible. It is then important to take large enough samples from each of these species, or to take a sample of several of these species, large enough to have reasonable power to demonstrate that *Bd* is truly absent if it is absent, or to estimate its prevalence with a reasonable degree of precision if it is present. Ideally, a at least 366 individuals, all belonging to species known to be susceptible to *Bd*, should be taken, and at samples should be taken from at least 35 individuals of each species sampled, allowing individual species to be examined with a reasonable level of confidence.

As discussed earlier, samples can be combined for PCR analysis. This is best done by the laboratory processing the samples, since combining samples in the field will inevitably increase the chance of cross-contamination and environmental contamination. If only partial samples are combined, or samples are combined after DNA extraction, then uncombined samples can be retained for further analysis. This strategy can substantially reduce the number of samples that must be processed, without compromising the precision of prevalence estimates. See http://www.ausvet.com.au/pprev/ for a prevalence calculator for pooled samples, and a more extensive discussion of this topic.

Established molecular labs currently conducting commercial PCR assays for *Bd*

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ADDITIONAL INFORMATION

Online database of literature related to *Bd*:
www.jcu.edu.au/school/phtm/PHTM/frogs/bibliog.htm

Online taxonomic database: Amphibian Species of the World
http://research.amnh.org/herpetology/amphibia/index.php

Additional information on amphibians and the amphibian crisis:
www.amphibians.org
www.amphibianark.org
www.amphibiaweb.org
www.globalamphibians.org
www.saveafrog.org

Amphibiaweb Ecuador
http://www.puce.edu.ec/zoologia/vertebrados/amphibiawebec/index.html

Costa Rican Amphibian Research Center
www.cramphibian.com

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