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# DETECTION OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN *ELEUTHERODACTYLUS FITZINGERI*: EFFECTS OF SKIN SAMPLE LOCATION AND HISTOLOGIC STAIN

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ABSTRACT: *Batrachochytrium dendrobatidis* is a fungal pathogen that has been implicated in amphibian declines worldwide. Histopathologic techniques have been used to diagnose the disease, but their sensitivity has not been determined. It is also unclear whether the probability of detection varies between skin samples derived from different body parts. We examined 24 Fitzinger's rainfrogs (*Eleutherodactylus fitzingeri*) with chytridiomycosis. This is a common frog species with a broad range and high abundance throughout most of Costa Rica. We sampled 12 different body parts from each animal, and alternated the staining between a routinely used stain (hematoxylin and eosin [H&E]), and a more fungus-specific stain (periodic acid–Schiff [PAS]). The pelvic patch and the innermost finger of the hand were consistently the best places to detect the disease, although significant differences were found only with the gular area, the abdomen, and toes four and five. We found more positive samples using PAS than using H&E in all body parts, although significant differences were detected only in samples derived from the pelvic patch. Using the best combination of factors (stain and body part) and animals with the lightest infections (to test the sensitivity of the technique), we calculated that at least 17 sections are needed in order to reach 95% confidence that a frog is or is not infected. We conclude that the choice of stain and body part can significantly alter estimates of prevalence of *B. dendrobatidis*.

*Key words:* Amphibian declines, Costa Rica, *Batrachochytrium dendrobatidis*, detection, histology, sensitivity, staining technique.

## INTRODUCTION

The chytrid fungus *Batrachochytrium dendrobatidis* has been implicated in amphibian declines around the world (Berger et al., 1998; Collins and Storfer, 2003; Daszak et al., 2003; La Marca et al., 2005; Lips et al., 2006; Pounds et al., 2006), and the low genetic variability (Morehouse et al., 2003) observed in *B. dendrobatidis* cultures worldwide supports the hypothesis that this is an emerging infectious disease (Berger et al., 1998; Daszak et al., 1999; Daszak et al., 2003; Lips et al., 2006). In Costa Rica, *B. dendrobatidis* has been found on dead or dying anurans (Lips et al., 2003) and on museum specimens collected prior to the population crashes (Pounds and Crump, 1994; Pounds et al., 1997; Lips, 1998, 1999).

Chytrid infections can be diagnosed by histopathologic techniques (Pessier et al., 1999; Berger et al., 2000), especially in cases of heavy infection. However, if light infections are common, a significant number of false negatives can be expected. For example, Davidson and collaborators (2003) acknowledge the fact that poor sensitivity could have influenced their analysis of chytrid infections in tiger salamanders, but did not attempt to quantify it.

Herpetologists often remove amphibian fingers for mark-recapture studies to estimate population sizes (Donnelly and Guyer, 1994), and recently this technique has been used to assess the presence of *B. dendrobatidis* in natural anuran populations (Berger et al., 1999). However, no information is available on the sensitivity of this approach. Problems can also be encountered with specificity when utilizing histopathologic techniques because chytrid zoospores can be confused with other cells, such as granulocytes. Olsen et al. (2004) developed a technique that is based on the idea that *B. dendrobatidis* only grows on keratinized skin, which should make detection more accurate when the pathogen is confirmed on keratin.

Histopathologic assays seem to be less sensitive than recently developed molecular assays (Boyle et al., 2004; Annis et al., 2004), and to improve histologic detection, Berger et al. (2002) developed a chytrid-specific inmunoperoxidase stain. However, not all laboratories have the ability to process antibody-based reactions, and histopatholgy is still widely used for diagnostics. Silver stains (Arrington, 1992), which are more specific to fungi, are not ideally suited to processing larger sample sizes because of time requirements. There is no published information on the comparison of routine histologic procedures with regard to their capacity to detect *B. dendrobatidis*.

The objective of this study was to compare the probability of detecting *B. dendrobatidis* in naturally infected Fitzinger's rainfrog (*Eleutherodactylus fitzingeri*) using two histologic techniques and skin samples derived from 12 different body parts.

## MATERIALS AND METHODS

Two routine stains were evaluated: hematoxylin and eosin (H&E) (Allen, 1992) and periodic acid–Schiff (PAS), which stains fungus glycogen, mucin, and some basal membranes magenta (Gaffney, 1994). Both stains are easily performed in the laboratory and are time-efficient when dealing with large sample sizes.

*Eleutherodactylus fitzingeri* was chosen because of its broad range, high abundance throughout Costa Rica (Savage, 2002) and previous observations of *B. dendrobatidis* infection in this species (Puschendorf, unpubl. data). During 2002 and 2003, we collected 99 specimens of *E. fitzingeri*; these were euthanized and preserved in formalin. An additional 237 specimens deposited in the herpetologic collection of the zoology museum of Universidad de Costa Rica were also analyzed. For each specimen an approximately 3-mm$^2$ patch of skin, at least 2.0 mm in length, was removed from the pelvic patch (PP), dehydrated, and embedded in paraffin. Two slides, each containing six ribbons of 4-μm sections, were stained, one with H&E, the other with PAS. Between each slide, 8 μm of the block was removed to avoid resampling sporangia. Three sections of skin (3 mm$^2$ with a minimum of 2 mm in length) from the pelvic patch (PP), abdomen (AB), and gular area (GU), along with the left hand and foot, were fixed in 10% neutral buffered formalin (Fig. 1). The samples were processed routinely and embedded in paraffin. The hand and foot were decalcified in a solution of ethylenediaminetetraacetic acid for 24 hr. We made six slides of each body part. With PP, AB, and GU, we placed six ribbons on each slide. Three samples of the hand and foot were placed on each slide.

The first, third, and fifth slides were stained with H&E. The second, fourth, and sixth were stained with PAS and examined for presence or absence of chytrid fungus. In the PP, AB, and GU slides, each section was individually examined and scored as infected or not. For the hand (F1–F4) and foot (T1–T5), a positive or negative value was given for each finger or toe. Because we were simulating a study in which fingers were removed for a mark-recapture study, only the digits were used to detect disease presence or absence; the reminder of the hand and foot were not considered. Detecting one sporangium was sufficient to classify a sample as infected. For PP, AB, and GU, we diagnosed 2,592 ribbons. We also evaluated 3,888 cross-sections of digits of 216 fingers for this disease. Mean prevalence of positive sections±standard error is reported.

We performed heterogeneity tests to assess whether the different data sets were comparable and could be pooled (Zar, 1999). Both H&E ($\chi^2$=1,314.7; 253 df; $P$<0.0001) and PAS ($\chi^2$ =1,213.8; 253 df; $P$<0.0001) were heterogeneous. Because of this, we used prevalence (positives/totals) data and applied the arcsine transformation before performing a repeated-measures analysis of variance to determine differences between detection rates between the two stains and the different body parts (Zar, 1999). To assess differences between and within the stains and body parts, we used post hoc homogeneous Tukey tests ($\alpha$=0.05).

## RESULTS

Differences in infection among body parts were significant ($F$=8.032; df= 11,253; $P$<0.0001). Although skin from PP (0.715±0.050) and F1 (0.787±0.054) were the most consistent places to find chytrid, they differed significantly only from GU (0.315±0.058), AB (0.412± 0.067), T4 (0.542±0.066), and T5 (0.491± 0.065) (Fig. 2).

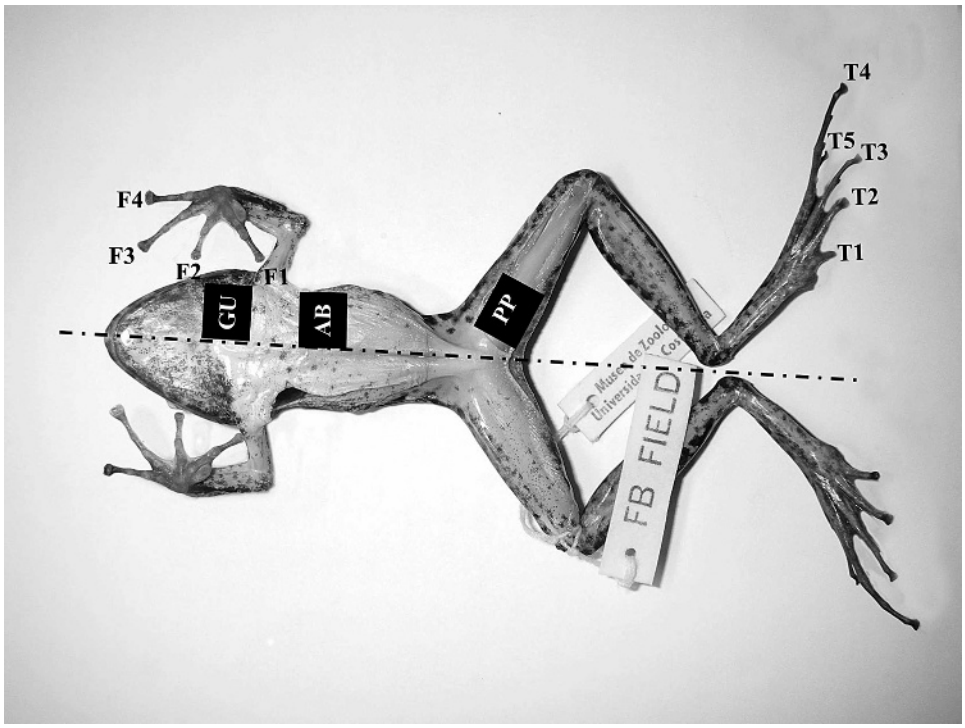


FIGURE 1. Body parts examined.

We found more samples infected with chytrid using PAS (0.637±0.025) than H&E (0.057±0.026) in all 12 body parts ($F$=25.506; df=1,23; $P$<0.0001). However, only PP (H&E 0.613±0.080; PAS 0.817±0.550) significantly differed between stains (Fig. 3).

We compared the effects of both body part and stain to determine which interactions would be the best and worst in detecting chytrid infection. Skin samples from PP (0.817±0.550) and F1 (0.792±0.078), both with the PAS stain, are the most effective in detecting infections. Samples from AB (H&E 0.377±0.092, PAS 0.447±0.097) and GU (H&E 0.262±0.075, PAS 0.368±0.090) were the worst places to look for the disease, using either stain (Fig. 3).

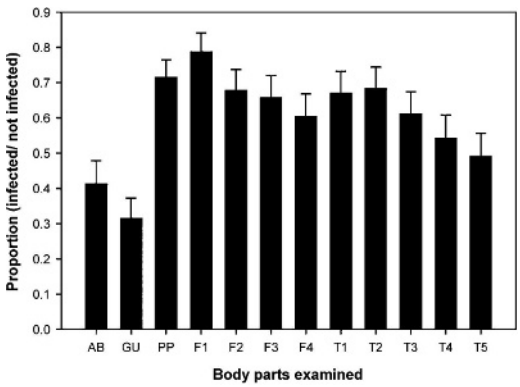


FIGURE 2. Average and standard error of infection in the different body parts.

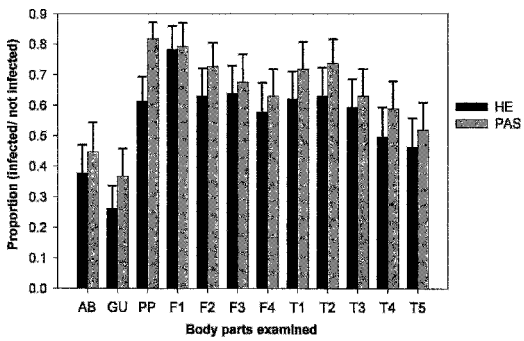


FIGURE 3. Average and standard error of infection in the different body parts using the H&E and PAS stains.

TABLE 1. Number of sections needed in order to achieve different confidence levels. The average infection of the 24 animals on the most powerful detection combination (PP×PAS), plus the six animals with the lightest PP infection is shown here. The probability that none of the sections examined were positive is also shown here. *n* equals the number of animals in the study with that infection rate.

| No. of sections examined | No. of positives/total no. of sections | | | |
|---|---|---|---|---|
| | 9/18 ($n=2$) | 7/18 ($n=2$) | 6/18 ($n=1$) | 3/18 ($n=1$) |
| 1 | 0.50000 | 0.61111 | 0.6667 | 0.833 |
| 2 | 0.25000 | 0.37346 | 0.4444 | 0.6944 |
| 3 | 0.12500 | 0.22822 | 0.2963 | 0.5787 |
| 4 | **0.0625** | 0.13947 | 0.1975 | 0.4823 |
| 5 | **0.0313** | 0.08523 | 0.1317 | 0.4019 |
| 6 | | **0.05209** | 0.0878 | 0.3349 |
| 7 | | **0.03183** | **0.0585** | 0.2791 |
| 8 | | | **0.0390** | 0.2326 |
| 9 | | | | 0.1938 |
| 10 | | | | 0.1615 |
| 11 | | | | 0.1346 |
| 12 | | | | 0.1122 |
| 13 | | | | 0.0935 |
| 14 | | | | 0.0779 |
| 15 | | | | 0.0649 |
| 16 | | | | **0.0541** |
| 17 | | | | **0.0451** |

Using the best skin sample location (PP) and stain (PAS), we used the binomial 95% confidence limit to calculate the probability of detection associated with the number of sections examined (Table 1). For this, we used results from six animals with light infections (chytrid detected in 3–9 of the 18 tissues previously examined). In some cases up to 17 sections are needed to reach a >95% confidence of detection.

## DISCUSSION

It is not surprising that skin samples from body sections in constant contact with the ground have a much higher chance of having chytridiomycosis than samples from other areas, such as the GU and AB. However, no systematic sampling has been done with other species. A similar pattern of infection was found in one *Hyalinobatrachium fleischmanni* specimen that was bred and died in captivity, indicating that this could be a shared infection process across species (Puschendorf and Kubicki, unpubl. data). Infections probably start in these areas. As the disease develops, they probably spread to sections of the GU and the AB. The PP is an extremely vascularized area in most anurans because of its major role in osmoregulation (Duellmann and Trueb, 1994). The skin here is constantly moistened and in contact with the ground. Removing skin in this area in live animals for disease surveys might not be feasible, but should be included in any scraping techniques using PCR to detect the disease.

In mark-recapture studies, it is advised to spare the thumb from being cut, because that could limit sexual behavior in males during amplexus (Duellman and Trueb, 1994). Most projects that have used fingers for analysis probably underestimate infection rates. For example, our data show that with the T5×H&E combination and 432 sections, only an average of 46% of the samples were found to be infected (Fig. 3). It is clear that even if the best combinations of factors are used, a few ribbons are not enough to determine whether a specimen is infected. A strong effort should be made before deciding that the fungus is absent.

Although PAS was always slightly better at detecting chytrid infections, our results show that it was significantly better at detecting the disease only in PP. Although H&E is more commonly used as a routine stain in diagnostic laboratories, we recommend the use of PAS for people with little experience in diagnosing the disease, such as students or herpetologists without backgrounds in pathology, and with an interest in knowing whether this pathogen occurs in their study site.

Although newer and more accurate genetic assays for the detection of fungal infections have been developed (Annis et al., 2004; Boyle et al., 2004), work on formalin-fixed museum specimens will

probably continue relying on histologic techniques (Schander and Halanych, 2003). Finding old museum specimens that are infected could become an impossible task because of the relatively small sample size of amphibians found in collections per collection event. If the abundance of *B. dendrobatidis* were lower, even more samples would be needed.

Another way of improving the detection power would be by cutting down the skin from the ventral side and rolling it up before taking a transversal sample of the roll of skin (Green, unpubl. data). This technique increases the sampled area greatly, but causes great damage to the specimen.

In conclusion, a large sample size, both in term of specimens and of sections per specimen, is necessary in order to minimize false negatives. The data presented here show that it will be especially hard to prove that *B. dendrobatidis* was not present at a site before the amphibians there disappeared. At least in the neotropics, old and systematic collections of sites seem to be very scarce.

In the tropics, highland sites, which have presented no evident declines documented so far, might hold the key in proving whether this pathogen is introduced or has always been around these sites, causing sporadic outbreaks in response to other triggering factors. Lips et al. (2006) show strong evidence that in Panama, *B. dendrobatidis* is an emerging infectious disease. Although the information provided here makes museum specimen surveys for this pathogen a more challenging task, it should not discourage researchers from doing so. It is a key effort to increase our knowledge on the evolution of this pathogen worldwide.

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