Abstract

DETECTION AND DISTRIBUTION OF THE AMPHIBIAN FUNGAL DISEASE CHYTRIDIOMYCOSIS IN PERUVIAN AMPHIBIANS

by

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Chytridiomycosis is an amphibian disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd;*Longcore et al. 1999). This disease has been identified by the Amphibian Conservation Action Plan as one of the main causal agents of worldwide amphibian declines and extinctions, and as the worst infectious disease ever recorded among vertebrates. Prior to this investigation, little was known about the prevalence of *Bd* across Peru, a country renowned for its high amphibian diversity and endemism. Here, I report the results of a sampling effort for *Bd* prevalence from the dry seasons of 2007 and 2008, which showed that among site *Bd* prevalence ranged from 0 to 25%. In this study, *Bd* was detected in 11 of 983 individuals sampled, and in nine out of 38 sites ranging in altitude from 96-3240 meters. I also discuss the implications of these results and suggest directions for further studies of *Bd* in Peru in order to better understand the ecology of this devastating disease and its effects on this biodiverse region.

In addition to investigating the prevalence of *Bd* throughout Peru, I also was interested in comparing and developing methods for the detection of *Bd*. Since the discovery of *Bd*, several methods have been utilized for detection; among these PCR from skin swabs is accepted as the best method due to its high sensitivity, non-invasiveness and ease of use. However, since PCR is a chemical reaction that requires a specific DNA template to proceed, this method relies upon both the presence of non-degraded DNA template and reaction components that do not inhibit the process. Here I present the results of experimental comparisons of techniques for sample preservation, DNA extraction, and PCR. My results show that the most advantageous techniques for a *Bd*-field study are swab preservation in 95% ethanol, DNA extraction with DNeasy, and an end-point PCR disease assay with BSA and Amplitaq Gold. I also recommend the use of PowerClean for samples with suspected PCR inhibitors. My hope is that these techniques will promote increases in sample collection and laboratory analyses for *Bd* research in developing countries where such activities are drastically needed.

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CHAPTER 1: BACKGROUND INFORMATION

What is *Bd*?

The chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter, *Bd*), has emerged as a novel pathogen afflicting amphibian populations worldwide (Fisher et al. 2009). It has been identified as a major factor driving amphibian declines and extinctions, making it one of the most destructive infectious diseases ever identified (Gascon et al. 2007). Declines and extinctions have been recorded from many different countries including: the United States (Briggs et al. 2005), Europe (Bosch and Martinez-Solano 2006), Central and South America (Lips et al. 2008) and Australia (Berger et al. 1998). The IUCN recently reported that 30 percent of all amphibians are at risk of extinction (Vié et al. 2009), making this the most critically endangered group of vertebrates in the world. In many cases, these declines are occurring in places that are not obviously affected by factors typically associated with decline, such as pollution and deforestation. Some sort of infectious agent is implicated in these cases, and the main culprit appears to be *Batrachochytrium dendrobatidis* (*Bd*), which causes the often lethal disease chytridiomycosis.

Chytridiomycosis was identified first by Berger et al. (1998) and then described by Longcore et al. (1999). Extensive population sampling has demonstrated that this disease can and does cause both dramatic declines of entire amphibian communities and local population extinctions (e.g., Lips et al. 2006). However, there are examples where *Bd* has infected populations, but dramatic declines have not occurred (e.g., Briggs et al. 2005, Puschendorf et al. 2006). Currently, it is unclear whether differences in the impact of the chytrid are caused by variation in environmental conditions (Kriger et al. 2007), ecological factors (Lips et al. 2003),

host immunity (Woodhams et al. 2006), differences among strains (Berger et al. 2005), or some combination of these factors.

Bd belongs to the basal fungal lineage Chytridiomycota which is comprised of mostly soil dwelling or aquatic detritivores (James et al. 2006). *Bd* is the only member of Chytridiomycota known to parasitize vertebrates (Longcore et al. 1999). Chytridiomycota is characterized by the presence of a mobile, flagellated zoospore stage (Longcore et al 1999). In culture, *Bd* can be grown easily on tryptone media and grows best at 17–25°C (Piotrowski et al. 2004). Other important abiotic conditions for growth in culture include humidity and pH. The life cycle begins with a motile zoospore which eventually encysts in the keratinized region of the host's epidermis, where it then develops into a zoosporangium (Piotrowski et al. 2004). Each zoospore produces a single zoosporangium that later divides mitotically to produce more zoospores (Berger et al. 2005b). After maturation, new zoospores are released into the environment via a discharge tube (Berger et al. 2005b). The entire life cycle in culture lasts four to five days at 22°C (Piotrowski et al. 2004). In tadpoles, *Bd* is restricted to keratinized mouthparts (Berger et al. 2000) and does not normally induce mortality (Marentelli et al. 2004, but see Blaustein et al. 2005). In juveniles and adults, *Bd* is dispersed across the epidermis, but tends to be especially concentrated in the feet and pelvic region (Berger et al. 2005c). In these individuals, the pathogen disrupts active electron transport across the skin (especially sodium) causing eventual death due to altered homeostasis (Voyles et al. 2009). Although the mechanism by which *Bd* disrupts electrolyte transport across the skin is unknown, Voyles et al. (2009) demonstrated that electrolyte transport across the skin was inhibited by as much as 50% in *Bd* infected individuals, which contributed to a 20-50% reduction in plasma potassium and sodium ion concentrations and lead to ultimate death by asystolic cardiac arrest.

Investigations in the field also have shown that a variety of environmental factors influence *Bd* virulence. Some of these include: altitude (Woodhams and Alford 2005, Brem and Lips 2008), temperature (Woodhams and Alford 2005), precipitation (Ron 2005, Fisher et al. 2009), and habitat type (Kriger and Hero 2007, Brem and Lips 2008). Additionally, various ecological and evolutionary factors associated with the host have been shown to dramatically affect pathogenicity: lifetime aquatic index (i.e., a measure of time spent associated with water, Lips et al. 2003), riparian reproduction (Kriger and Hero 2007, Brem and Lips 2008), endemicity (Bielby et al. 2008, Smith et al. 2009), and evolutionary history (Corey and Waite 2008). Using *Bd* distribution data and many of the factors listed above, ecological niche modeling has been utilized in various ways to make broadscale predictions concerning the localities that are highly susceptible to *Bd*-related declines (Ron 2005, Bielby et al. 2008, Rödder et al. 2009). Many of these models predict that amphibians living tropical and subtropical montane habitats are the most vulnerable to chytridiomycosis declines.

Origin of *Bd*

There is substantial debate concerning the origin of *Bd* as a lethal pathogen. Some researchers have proposed that *Bd* is a recently emerged pathogen that is sweeping through amphibian populations ("novel pathogen hypothesis" of Berger et al. 1998, Skerratt et al. 2007). A variety of evidence supports this hypothesis. For example, in both Central America and Australia, researchers have monitored areas predicted to be affected by an advancing wave of chytrid infection (Berger et al. 1998, Lips et al. 2006). Samples taken during early monitoring did not reveal the presence of the chytrid. This was followed by a wave of infections affecting virtually all amphibians, resulting in massive declines and local extinctions. At the genetic level, Morehouse et al. (2003) found extremely low levels of genetic variation among *Bd* isolates from Africa, Australia, North America and Panama, further supporting the hypothesis that *Bd* is novel, emerging pathogen. Several subsequent surveys have found similar results (Morgan et al. 2007, James et al. 2009). Although there have been many long-term studies of amphibian populations since the 1900's, there were no records of mass mortality caused by disease until the late 1970's when the first *Bd* epidemics were reported (Barinaga 1990). However, inspections of museum specimens have detected *Bd* in skin cross-sections from as long ago as 1902 in Japan (Goka et al. 2006) and 1938 in Africa (Weldon et al. 2004). It has been proposed that a novel, pathogenic form of the chytrid was spread globally through the exportation of a host species, the African Clawed Frog (*Xenopus laevis*), in the 1960's (Weldon et al. 2004). This species was exported across the world in massive quantities for use in human pregnancy tests. Recently, a comparison of 20 *Bd* genomes from multiple continents found three distinct lineages of the fungus which differ in virulence and other morphological traits (Farrer et al. 2011). They provide evidence that the hypervirulent lineage observed worldwide may have arisen from a recombination event from

two less virulent lineages which likely came into contact from the global trade of amphibians (Farrer et al. 2011).

In contrast to the novel pathogen hypothesis, the "endemic pathogen hypothesis" (Rachowicz et al. 2005, Pounds et al. 2006) proposes that *Bd* was present in the environment (in a saprobic or sporitic form) before the outbreaks, but that some kind of environmental change caused the fungus to become pathogenic. There is some evidence for an inactive, non-pathogenic form of *Bd* (Di Rosa et al. 2007), but this has yet to be verified Evidence of correlations between warming trends and *Bd*-related declines also are consistent with the endemic pathogen hypothesis (e.g., Pounds et al. 2006, Alford et al. 2007).

Since the discovery of *Bd* over a decade ago, considerable progress has been made to ascertain the mechanisms of global dispersal and the origin of this pathogen. The research of Morehouse et al. (2003), Morgan et al. (2007), James et al (2009), and most recently Farrer et al. (2011) has been especially insightful at contributing to the understanding of the origin of this disease. Yet, many important questions remain to be answered--most pressing the identification of the geographic origin of *Bd*. Here, I try to address a small part of this enigma by investigating the prevalence of this disease in a biodiverse and understudied region. In the future, I plan to use the many of the skills and samples obtained from this research to continue to address this question using phylogeographic methods.

Bd Worldwide Distribution

Bd is has been found on all continents that amphibians are known to occur (Fisher et al. 2009). Despite this, sampling for *Bd* has been somewhat biased in that the majority of studies that have been conducted to date have taken place in developed countries (i.e., United States, Europe, Australia, Global *Bd*-Mapping Project 2012). Unfortunately, amphibian diversity and endemism often are highest in developing regions (Figure 1, IUCN Red List 2008) meaning that a high proportion of global amphibian diversity may be threatened by *Bd* and we may not even be aware of it. The scarcity of *Bd* studies in developing countries likely occurs because of a combination of factors including the general lack of funding, absence of molecular facilities, shortage of trained investigators, and ease of access to sampling sites. Of these, the inability for researchers to perform the PCR diagnostic procedure 'in-house' is probably the biggest contributor to the scarcity of research in these countries. This is unfortunate due to the high value of information on *Bd* prevalence from these regions. and because the PCR disease assay can be performed by individuals after minimal training investments and moderate monetary investment. A major component of my dissertation work has been to develop a *Bd* PCR assay is not only reliable, but that can be performed by individuals with a minimal training investment and at facilities with only a moderate monetary investment. I will discuss the development and substantiation of this assay in Chapters 3 and 4 of this document.

Bd in South America and Peru

Our knowledge of the history of *Bd* in South America begins in Ecuador where it was first detected in 1980 (Ron and Merino-Viteri 2000). Since then, *Bd* has been detected in most South American countries where samples have been analysed (Carnaval et al. 2006). Intensive monitoring efforts in Ecuador have documented the effects of *Bd* infection on montane endemic populations of frogs in the genus *Atelopus*, where it has caused widespread extinction at both the population and species level (Ron and Merino-Vitteri 2000, La Marca et al. 2005). Other Ecuadorian species also have been affected, as documented by intensive monitoring at seven different sites (Bustamante et al. 2005).

In Brazil, the first record of *Bd* (1981, Carnaval et al. 2006) comes from the Atlantic Forest region. Interestingly, this record coincides with several historical reports in this area of unexplained declines (Carnaval et al. 2006). *Bd* has been well studied in this region; to date, 21 species have been found to be infected (Carnaval et al. 2006, Toledo et al. 2006). Other regions throughout the country remain under-sampled including areas that have been predicted to be suitable for disease establishment by niche modelling (Ron et al. 2005) such as the Cerrado and Pantanal regions.

In contrast to Ecuador and Brazil, comparatively little effort has been spent monitoring amphibian populations for the effect of *Bd* infection in Peru and other South American countries (Lips et al. 2005). Yet, Ron et al. (2005) identified the eastern slopes of the Andes in Peru and Bolivia (through niche modeling) as one of the new world regions with highest suitability for *Bd* establishment. The cordilleras that make up the transition zone between the Andes Mountains and Amazonian lowlands are one of the most species rich regions on earth (Fjeldsa and Rahbek

2006). Species richness and regional endemicity are particularly high for amphibians in this area (Duellman 1982). In fact, the area is unrivalled in amphibian biodiversity (Duellman 1999). Peru is situated along the majority of the Andean massif, with vast tracts of transition zone cordilleras spanning the gap between the Andean highlands and the Amazonian lowlands. Such habitat variation promotes biodiversity and allows Peru to be ranked fourth in worldwide amphibian biodiversity survey (Vié et al. 2009). The emergence of *Bd* in this region potentially poses a grave risk to this biodiversity.

The first record of *Bd* in Peru came from several dead *Atelopus patazensis* collected in 1999 (Venegas et al. 2008) this species is currently listed as critically endangered by the IUCN due to the combined effects of *Bd* and water pollution (IUCN 2011). Reliable data are not available on the status of other *Atelopus* species in Peru (Venegas et al. 2008, von May et al. 2008), though many are known to be declining in other countries (La Marca et al. 2005). Population surveys for *A. pulcher* in northern Peru have not detected any adults in localities where they were once abundant; one of the last individuals seen was found dead and later tested *Bd*-positive (Lötters et al. 2005). Another study in northern Peru, failed to detect *Bd* in 23 individuals tested using histopathological analysis (Enciso et al. 2008). In southern Peru, the first record of *Bd* came from three of four *Telmatobius marmoratus* collected in 2002 in the Cordillera Vilcanota (Seimon et al. 2005). A later study in this region reported a range expansion of *Bd* to the highest altitude yet recorded (5348 m). Seimon et al. (2007) posit that this expansion may be due to the recent deglaciation and subsequent host population expansion in the region. Additionally, it appears *Bd* may be contributing to declines in *T. marmoratus*, one of the two host species investigated in this study (Seimon et al. 2007). Most recently, Catenazzi et al. (2011) discovered dramatic declines in montane amphibian species richness and abundance in

southern Peru between surveys from 1999 and 2008-2009. They attributed these declines to chytridiomycosis rather than habitat loss because *Bd* is widespread in the region and declines occurred within the well-protected zone of Manu National Park (Catenazzi et al. 2011).

Detection of *Bd*

Since the discovery of *Bd* in 1998 (Berger et al. 1998), several methods have been utilized for its detection from amphibian hosts: these methods include clinical symptoms and mortality, histology, end-point Polymerase Chain Reaction (PCR), and quantitative PCR (qPCR). PCR is a technique that uses specific oligonucleotide primers to amplify a targeted DNA molecule across many orders of magnitude. Mechanistically, end-point PCR differs from qPCR in that the end results are determined by scoring for the presence of bands using gel electrophoresis, whereas, qPCR utilizes an apparatus that detects increases in the fluorescence of a reporter dye, which either intercalates to double stranded DNA or hybridizes to specific complementary DNA targets. Measuring fluorescence to detect targeted DNA with qPCR also has the added advantage of allowing quantitation of accumulating product, which can be used estimate original sample concentration. In contrast, end-point PCR cannot reliably estimate sample DNA concentration and is normally only used for detection purposes or DNA sequencing studies.

Today PCR of amphibian skin swabs (especially qPCR) is the most widely used method for *Bd* detection (Hyatt et al. 2007). Both end-point and quantitative PCR have many advantages over previous methods. First, PCR methods are far more sensitive (likely to diagnose the disease when present at low levels) than other published methods, and this is especially so within the early stages of *Bd* infection when diagnosis by clinical symptoms or histology is limited if not impossible (Boyle et al. 2004, Kriger et al. 2006b). This is because most amphibians do not display clinical symptoms until the latter stages of infection (i.e., around 10-21 days, Rosenblum et al. 2009). In addition, the primary infection may be patchy or limited to one body region making detection with histology a game of chance (Boyle et al. 2004, Kriger et al. 2006). PCR is

advantageous here in both cases, because it is able to detect extremely low levels of the disease (i.e., 0.1 zoospore equivalents, Boyle et al. 2004), which allows diagnosis as early as day three (Rosenblum et al. 2009). PCR of skin swabs also has the added advantage of covering the entire surface of the animal thus increasing likelihood of detection, which is advantageous the infection distribution is patchy. Secondly, PCR has greater specificity (ability to correctly diagnosis the disease) than other methods (Hyatt et al. 2007). This is especially true when compared to diagnoses based upon clinical symptoms or mortality events, as *Bd* cannot be readily distinguished from other diseases due to its similarity in symptoms to some viral diseases and bacterial infections (e.g., red-leg syndrome, Densmore and Green 2007). *Bd* diagnosis with histology is more reliable than clinical diagnosis. Most authors (e.g., Hyatt et al. 2007) agree however, that histology is still limited in comparison to PCR because it relies upon tissues that are relatively intact and also upon the skill of the observer at distinguishing *Bd* cellular structures from that of the host. In contrast to the aforementioned methods, *Bd* diagnosis with PCR is "black and white". This is especially true with end-point PCR where the observer is simply scoring for the presence/absence of a 300 base pair band on a gel to diagnose the disease. PCR and clinical diagnosis have comparable invasiveness as a procedure; both require that the amphibian be handled for a brief period of time for examination or swabbing in the case of PCR. In comparison, histology necessitates tissue collection or sacrifice of the entire animal, which can be problematic especially when working with rare or threatened species where the sacrifice of individuals may not be acceptable. Lastly, PCR differs from histology in the ease of the procedure. Performing histological examinations requires special training and experience; considerable time must be spent collecting tissue sections from various regions of the animals' body to increase the likelihood of a correct diagnosis (Boyle et al. 2004). In contrast, PCR

techniques can readily be taught to undergraduates with a small time investment and have the additional benefit of allowing the researcher to include positive and negative reaction controls.

Management of *Bd*

Various strategies for managing *Bd* are currently being employed (e.g., preventing further spread, establishing captive assurance colonies); however, it is still too early to evaluate their long-term effectiveness (Woodhams et al. 2011). The Amphibian Conservation Action Plan (Gascon et al. 2007) emphasized that a significant proportion of their proposed budget should be spent on efforts related to *Bd* (i.e., 6% of funding should be focused on research and management of emerging infectious diseases with a particular emphasis on *Bd*, 10% on establishing captive breeding programs, and 1% on developing reintroduction programs, Gascon et al. 2007). A recent review by Woodhams et al. (2011) stressed the importance of taking an epidemiologically-informed approach to *Bd* management. Such an approach would employ site-specific strategies on host immunity, local strain virulence, and climate, and would emphasize disease mitigation rather than eradication (Woodhams et al. 2011). The authors recommend using a three-pronged approach for *Bd* management consisting of: identifying mechanisms of disease suppression, parameterizing epizootiological models of disease and population dynamics for testing under semi-natural conditions, and performing adaptive management in field trials with natural populations (Woodhams et al. 2011).

Approaches such as those discussed in Woodhams et al. (2011) are a great "first step" towards getting *Bd* management 'off the ground'; however, before many of these projects can be initiated, a lot more research needs to be directed towards population surveillance. As mentioned previously, most amphibian populations in South America, the amphibian biodiversity center of the world, lack population monitoring altogether. Other amphibian biodiversity host spots such as South East Asia are even less understood (Vié et al. 2009). Until more efforts are invested in population surveillance, we will continue to loose amphibian populations at an alarming rate

from factors such as *Bd* and habitat loss without even being aware that they were declining in the first place. The study discussed in Chapter 2 of this document, was the first to evaluate the *Bd* status of amphibians across Peru and I believe that it is a great "first step" towards protecting these vulnerable animals; however, it is only a "first step" in a long road to conserving amphibians. One of my main research goals is to illustrate the importance of continued investigation of *Bd* in these understudied, biodiverse regions. Most importantly, I hope to reveal the value of investing in long term population monitoring, which I believe cannot come about unless there are major with changes in science and policy first.

Goals of Dissertation

The goals of my research were to investigate the prevalence of *Bd* across Peru and to optimize and compare methods for detection of *Bd* from field samples. The remainder of this dissertation is divided into four chapters. In Chapter 2, I discuss the results of my sampling effort for *Bd* prevalence in Peru, which was conducted during the dry seasons of 2007 and 2008. I also discuss some of the implications of the results of this investigation and directions for further studies of *Bd* in Peru. In Chapter 3, I review the results from my laboratory investigation on techniques for field sampling of *Bd*. In this study, I optimized and compared methods for field preservation of swabs, DNA extraction, and *Bd* detection. In Chapter 4 I discuss the results of my research on methods for minimizing the effect of PCR inhibitors in the *Bd* assay. Lastly, in Chapter 5, I summarize the major results of this dissertation and discuss the intellectual merits and broader impacts of this research.

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Figures

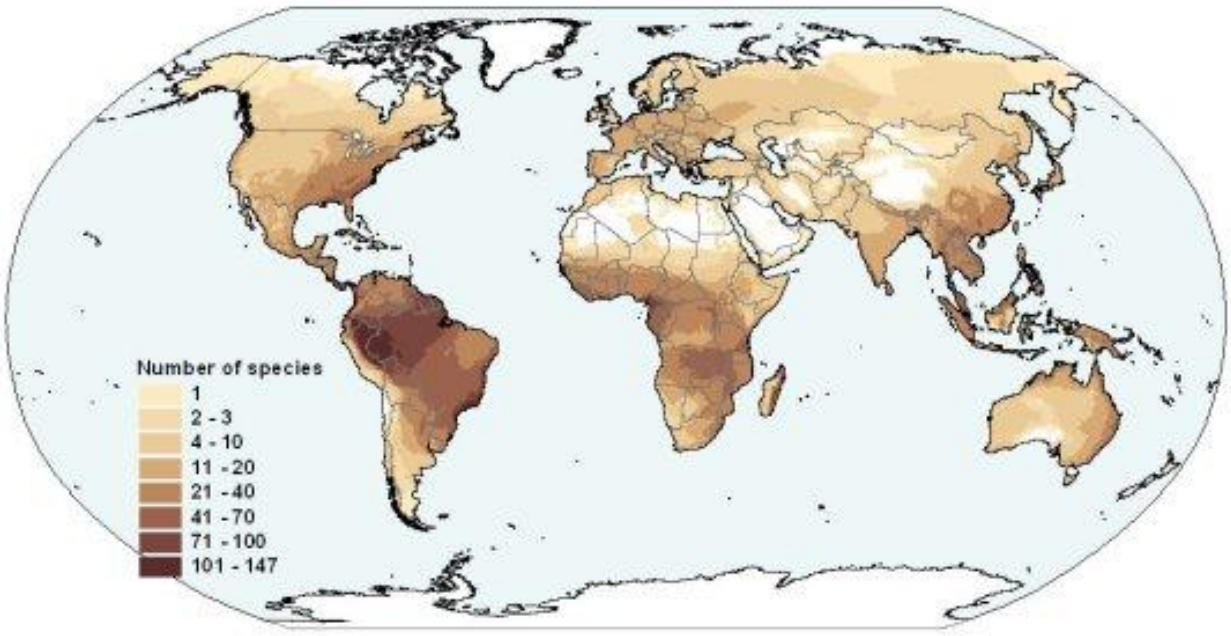

Figure 1. Global diversity of amphibian species. Reprinted from the IUCN Red List (Accessed: May 25, 2012.

CHAPTER 2: *BATRACHOCHYTRIUM DENDROBATIDIS* IN PERU*

Abstract

Chytridiomycosis is an amphibian disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). This disease has been identified as one of the main causal agents of worldwide amphibian declines and extinctions, and as the worst infectious disease ever recorded among vertebrates by the Amphibian Conservation Action Plan. Prior to this investigation, very little was known about the prevalence of this disease across Peru, a country renowned for its high amphibian diversity and endemism. Our sampling effort from the dry seasons of 2007 and 2008 show that *Bd* is widely distributed throughout Peru, with among site prevalence ranging from 0 to 25%. *Bd* was detected in 11 of 983 individuals sampled, and in 9 out of 38 sites ranging in altitude from 96-3240 meters. We detected *Bd* in 11 species of anurans belonging to 14 families. The family with the highest number of *Bd*-positive individuals was Hylidae with four positive samples, all from different species. The disease also was detected in one of two *Telmatobius marmoratus* purchased at a market in Cusco where they were being sold for human consumption further supporting the idea that human transport of amphibians is facilitating the dispersal of this *Bd*. We strongly recommend further studies of *Bd* in Peru in order to better understand the ecology of this devastating disease and its effects on this biodiverse region.

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Introduction

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has been well-studied in Australia, North and Central America, and Europe, but relatively little information is available concerning the status of this pathogen in South America. This is especially disturbing given that South America contains the highest diversity of amphibian species in the world (Vié et al. 2009).

In Peru, sampling for *Bd* has been especially sparse, with only six studies in localized regions conducted to date (Table 3). The first record of *Bd* in Peru came from several dead *Atelopus patazensis* collected in 1999 (Venegas et al. 2008). Although recent surveys have shown that this species continues to persist, it is currently listed as critically endangered by the IUCN due to the combined effects of *Bd* and water pollution (IUCN 2011). Reliable data are not available on the status of other *Atelopus* species in Peru, though many appear to be declining (Venegas et al. 2008, von May et al. 2008), a trend seen throughout the range of this genus (La Marca et al. 2005). Population surveys for *A. pulcher* in northern Peru have not detected any adults in localities where they were once abundant; one of the last individuals seen was found dead and later tested *Bd*-positive (Lötters et al. 2005). Another study in northern Peru failed to detect *Bd* in 23 individuals tested using histopathological analysis (Enciso et al. 2008). In southern Peru, the first record of *Bd* came from three of four *Telmatobius marmoratus* collected in 2002 in the Cordillera Vilcanota (Seimon et al. 2005). A later study in this region reported a range expansion in *Bd* to the highest altitude yet recorded (5348 m), which Seimon et al. (2007) posit may be due to the recent deglaciation and subsequent host population expansion in the region. Additionally, it appears *Bd* may be contributing to declines in one of the two host species investigated (Seimon et al. 2007). Most recently, Catenazzi et al. (2011) discovered dramatic

declines in montane amphibian species richness and abundance in southern Peru between surveys from 1999 and 2008-2009. They attributed these declines to chytridiomycosis rather than habitat loss because *Bd* is widespread in the region and declines occurred within the well-protected zone of Manu National Park (Catenazzi et al. 2011).

Recently, it has been shown that the global trade of amphibians is a major contributor to the worldwide dispersal of *Bd* (Fisher and Garner 2007, Farrer et al. 2011). In Peru, trade of amphibians is fairly common, especially in large cities such as Lima and Cusco where Andean frogs (predominantly *Telmatobius* and *Batrachophrynus* species) are frequently sold live in restaurants and markets as a protein source or for their perceived medicinal properties (Angulo et al. 2008). Investigations have shown that many of these frogs are *Bd*-positive (Catenazzi et al. 2010), indicating that they are likely contributing to spread of the disease throughout Peru as they are moved from capture sites to markets.

The aim of our investigation was to conduct a systematic survey of the current distribution of *Bd* in amphibian populations throughout Peru. Our main goal was to obtain a "snapshot" of the current distribution of the infection to aid in planning future research and management of the disease.

Materials and Methods

Field surveys of *Bd* prevalence were conducted during June–August of 2007 and May–July of 2008, coinciding with the dry season. We surveyed 39 sites along the eastern slopes of the Andes, providing a collection of samples along both latitudinal (3.68572°S, 73.28350°W to 13.17956°S, 71.60561°W) and altitudinal gradients (90–3240 m; Figure 2). Coordinates of each locality were determined using a portable GPS unit (Garmin *E*trex Vista, Olathe, KS). Sites were surveyed using the visual encounter technique (Lips et al. 2001). For each site, two surveyors performed a time-constrained survey for four hours. The primary goal of each survey was to capture as many individuals and species as possible. During each survey, all amphibians encountered were captured by hand and a new plastic bag was used each time to eliminate the possibility of cross contamination between individuals. For ease of species identification, only post-metamorphic individuals were sampled. After capture, all specimens were kept in individual plastic bags and stored in a cool place until each transect was completed. Appropriate measures were taken to prevent cross-contamination between sites (i.e., disinfection of equipment and footwear between sites, clean clothing, etc.).

During processing, a new pair of latex gloves was worn for each individual, and all equipment was disinfected between animals. Processing consisted of species identification, clinical examination for abnormalities (i.e., retained shed skin, reddening of skin), and collection of digital photographs of the dorsum and venter. The epidermis was swabbed using dry sterile rayon swabs (Medical Wire and Equipment, Durham, NC). Swabbing consisted of running a rayon swab 10 times over the dorsum, both sides, venter, undersides of each thigh, and five times on the underside of each foot, for a total of 80 swab runs per animal (Kriger et al. 2006). After processing, all specimens were released within two meters of their capture site (with the

exception of the frogs purchased at the San Pedro Market which were euthanized after processing). Swabs were preserved at room temperature in individually labeled vials of 95% ethanol.

DNA extractions for swabs were performed in the winter of 2008. The samples from 2007 were extracted in 2008 due to a delay in obtaining permits. During this time, these samples were stored at the Museum of Natural History in Lima at room temperature. Upon arrival in the US, all samples were immediately placed at 5°C until DNA extraction. DNA extraction was performed using the PrepMan Ultra Sample Preparation Reagent (ABI), following methods outlined in Boyle et al. (2004). Samples then were analyzed using a highly sensitive endpoint PCR *Bd* assay (minimum sensitivity of 0.0016 zoospores) developed in our laboratory (described in Chapter 3), which uses *Bd*-specific primers developed by Annis et al. (2004; *Bd*1a: 5'-CAGTGTGCCATATGTCACG-3', *Bd*2a: 5'-CATGGTTCATATCTGTCCAG-3'). The reaction recipe was: 1.25 µl of DNA Gold Buffer (10x, ABI), 1.0 µl of $MgCl_2$ (16.7 µM), 2.4 µl of GeneAmp dNTP mix with dTTP (1000 µM, ABI), 0.5 µl of forward and reverse primers (5 µM, Invitrogen), 0.06 µl of AmpliTaq Gold with GeneAmp (5 units/µl, ABI), 4.29 µl of DNA grade water (Fisher Scientific, Pittsburg, PA), 2.0 µl of sample for a total reaction volume of 12 µl. Reactions were loaded into a 96-well plate and placed in the PTC-200 Thermal Cycler (ABI). Initially all samples were run in duplicate (i.e., two separate PCR runs/sample/plate) along with positive (previously amplified *Bd* DNA from culture) and negative controls (PCR master mix and H_20). Inhibition controls were not used. We used the thermal cycler program: 1) 5 minutes at 95°C, 2) 45 seconds at 93°C, 3) 45 seconds at 60°C, 4) 1 minute at 72°C, 5) repeat steps # 2-4 for 44 more times, and 6) 10 minutes at 72°C. After thermal cycling, 8 µl of the resulting product was mixed with 1 µl of 6x Apex loading dye (Genesee) and loaded into a gel (8 ml of 1x TBE

Buffer, 1.2 g of Apex agarose, Genesee). Additionally, one well per row was loaded with 100 bp Promega DNA ladder (Fisher Scientific), so that the size of the PCR amplicons could be estimated. Gels then were immersed in 1x TBE Buffer and run at 120 volts for approximately 40 minutes, after which gels were examined and photographed using a UV viewing apparatus. The number of true positives and false negatives was determined by scoring the presence/absence of 300 bp bands on electrophoresis gels. For ambiguous samples, further PCR analyses were conducted. The data for each locality were summarized in terms of *Bd* prevalence (total number of infected individuals/total number of individuals sampled).

Results

Over a two-year period, a total of 983 amphibian skin swabs were collected from 39 sites throughout Peru. We collected samples from 36 genera of amphibians belonging to 14 families (Table 2, Figure 2). The results of our PCR assay showed that 11 of 983 individuals sampled were positive for *Bd* (overall prevalence = 1.0%, Table 1). *Bd* was detected in amphibians at nine of 39 sites across a broad range of altitudes (96–3240 m, Figure 2). Among-site prevalence ranged from 0 to 25% (Table 1, only results from natural populations were used in this analysis, therefore results from San Pedro market were excluded). *Bd* was detected in 11 species of anurans from seven families (Table 2).

The majority of *Bd*-positive individuals had reproductive modes associated with permanent bodies of water (6/11, *Allobates marchesiansus, Hypsiboas melanopleura, Hyloscirtus c.f. phyllognatus, Hyloxalus shuar, Osteocephalus buckleyi, Telmatobius c.f. marmoratus*) and/or had an aquatic tadpole stage (9/11, *A. marchesiansus, Engystomops petersi, Hypsiboas melanopleura, Hyloscirtus c.f. phyllognatus, Hyloxalus shuar, Leptodactylus petersii, O. buckleyi, Scinax garbei, T. c.f. marmoratus*). Five of the 11 *Bd*-positive individuals are known to utilize streams for reproduction *(Allobates marchesiansus, Hyloscirtus c.f. phyllognatus, Hyloxalus shuar, O. buckleyi, T. c.f. marmoratus).* The family with the highest number of infected individuals was Hylidae with four infected individuals (Table 2). *Bd* was not detected in any of the 23 *Atelopus* that we sampled (three *A. pulcher*, 20 *A. c.f. andinus*). One of the *Bd*-positive samples came from two *Telmatobius marmoratus* purchased at San Pedro Market in central Cusco (of unknown origin) where they were being sold for human consumption. Obvious clinical abnormalities consistent with possible signs of the disease chytridiomycosis were

detected in only three of 983 individuals (all *Gastrotheca excubitor*), which had retained shed skin on their toe pads. One of these three individuals tested positive for *Bd* with the PCR assay.

Discussion

The results of this and previous studies indicate that *Bd* is widespread throughout Peru, and from our limited data it appears that *Bd* presence may not be as tightly linked with altitude as suggested by others (Figure 2, Bielby et al. 2008, Brem and Lips 2008, Lips et al. 2008). This adds to the growing body evidence (Kriger and Hero 2008, Walker et al. 2010) that altitude may not have as strong an influence on disease prevalence as originally believed.

In this study, the majority of *Bd*-positive individuals had reproductive modes associated with permanent bodies of water and/or an aquatic tadpole stage, characteristics that have been shown to be associated with high transmission and prevalence of chytridiomycosis (Lips et al. 2003, Kriger and Hero 2007). Of these species, five are known to utilize streams for reproduction, a characteristic associated with susceptibility and declines in other regions (Lips et al. 2003, Kriger and Hero 2007, Catenazzi et al. 2011). There was a greater frequency of *Bd*-positive individuals in the family Hylidae. Other researchers have reported a higher frequency of chytridiomycosis in this family (e.g., Stuart et al. 2004), which may be linked to reproductive mode (Lips et al. 2003) and/or evolutionary history (Corey and Waite 2008). Contrary to our expectations, we did not detect *Bd* in any of the *Atelopus* that we sampled. Although this is the first time that a population of *A. c.f. andinus* has been sampled for *Bd*, this disease has been previously detected in other Peruvian *Atelopus* (*A. pulcher*: Lötters et al. 2005, *A. patazensis:* Venegas et al. 2008). Our data showing *Bd* in one of two *Telmatobius marmoratus* tested from the San Pedro market further supports the idea suggested by Catenazzi et al. (2010), that the trade of amphibians in Peru may contribute to the dispersal of *Bd* throughout the country.

The *Bd* prevalence we found among sites was lower than we expected, especially in mid-elevation regions where previous studies in other countries have reported high disease prevalence

(e.g., Sánchez et al. 2008). Other studies in Peru have reported among-site prevalence ranging from 0 to 100% (Table 3). In fact, Catenazzi et al. (2011) reported *Bd* prevalence as high 64–100% (in *T. marmoratus*) from some of the same sites that we visited in the Department of Cusco. There are several possible reasons for this. First, our surveys were conducted in the dry season when climatic conditions may be less conducive to disease transmission and progression (but see Burrowes et al. 2004). Second, our within-site sample sizes may not have been large enough to detect the disease (95% CI=26 ± 29). Statistical modeling has shown that at least 30 individuals need to be swabbed per locality in order to detect at least one positive individual at a site if the disease prevalence is 10% (Cannon and Roe 1982). As it was not always possible for us to collect 30 individuals per locality due to time limitations, this may explain the lower frequencies we observed. Lastly, although we know that the *Bd* assay that we used is equally sensitive to *Bd* detection techniques published by others (discussed in Chapter 3), another possibility for our lower number of *Bd* detections may be due to the presence of PCR inhibitors that have been known to contribute to incorrect reporting of false negatives (Garland et al. 2009). Another interesting observation from this investigation is that only three of 983 individuals had any obvious clinical abnormalities consistent with the disease chytridiomycosis, and we did not observe any die-offs or moribund individuals. This is may be because chytridiomycosis is difficult to detect by clinical examination (Green et al. 2002, Kriger et al. 2006), but may also indicate that the infection is not progressing to an advanced state, possibly due to host immunity or low virulence (especially if *Bd* has been present in Peru for long enough for adaptation to occur). Unfortunately, we are unable to distinguish between these possibilities with our limited data.

Although the results of this and previous studies demonstrate that *Bd* is widespread in South America (e.g., Brazil: Carnaval et al. 2006, Venezuela: Sánchez et al. 2008, Argentina: Barrionuevo and Ponssa 2008), the consequences of this disease are still relatively unknown due to the near complete absence of general population monitoring and *Bd* studies in these countries. In Peru, significant declines have been reported in multiple high-elevation species in southern Peru (Catenazzi et al. 2011), and several *Atelopus* and *Telmatobius* species are believed to already be extinct (Venegas et al. 2008, von May et al. 2008). Peru contains some of the highest amphibian diversity on record and the loss of this diversity could have severe consequences. This makes the lack of knowledge on *Bd* and population status of Peru's amphibians especially alarming.

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Tables and Figures

Table 1. Summary of amphibian study sites in Peru sampled for *Batrachochytrium dendrobatidis (Bd)*. Surveys were performed during the dry seasons (June–August) 2007 and (May–July) 2008.

Locality	Latitude	Longitude	Altitude (m)	N	*Bd* Positive	Prevalence
2007						
Ahuashiyacu	-6.4578	-76.30843	722	15	2	0.13
Allpahuayo Mishana	-3.75067	-73.28228	102	1	0	0.00
Bocatoma	-6.4585	-76.34968	426	43	0	0.00
Chacos Valley and Llamaquizú	-10.61583	-75.35056	1800-2100	4	1	0.25
Chazuta	-6.5692	-76.12622	235-355	42	0	0.00
Huallaga River	-6.58899	-75.91939	171-232	36	0	0.00
Iquitos Km 23	-3.9586	-73.375	149	37	0	0.00
Iquitos Km 71	-3.75067	-73.33228	122	15	0	0.00
Lamas	-6.38493	-76.51527	645	1	0	0.00
Nanay River	-3.68572	-73.2835	96	17	1	0.06
Pampa Hermosa	-10.9925	-75.43278	1200-1550	10	0	0.00
Pond near Tunnel	-6.43118	-76.30882	846	16	0	0.00
Pongo de Cainarachi	-6.29388	-76.23598	190-220	58	0	0.00
San Jose	-6.4194	-76.2901	470-589	58	0	0.00
Santa Rosa	-5.4406	-78.55473	1234-1270	11	1	0.09
Sapasoa	-6.89635	-76.82817	315-416	32	0	0.00
Sauce	-6.72453	-76.25318	622	5	0	0.00
Seco River	-8.59656	-76.08714	771	20	0	0.00
Tahuayo	-4.17703	-73.15365	115	26	0	0.00
2008						
Aguaytia	-9.05734	-75.66543	429	6	0	0.00
Chacos Valley and Llamaquizú	-10.61583	-75.35056	1800-2100	2	0	0.00
CICRA Station	-12.56861	-70.09917	250-270	83	1	0.01
Cueva de las Lechuzas	-9.32867	-76.02715	656	19	1	0.05
Huampal	-10.18825	-75.57519	968	6	0	0.00
Ivochote	-12.46317	-72.96740	471-481	32	0	0.00
Lake Milagros	-9.14174	-75.99635	671-691	17	0	0.00
Lake Yarinachocha	-8.32561	-74.59022	188	29	0	0.00
Manu Learning Center	-12.78926	-71.39175	463	51	2	0.04
Oxapampa	-10.54556	-75.35835	2355	12	0	0.00
Pilcopata	-12.90997	-71.42281	480-525	49	0	0.00
Pozuzo	-10.04908	-75.54059	692	10	0	0.00
Puente Maranura	-12.96498	-72.66568	1068	22	0	0.00
Puerto Bermudez	-10.27102	-74.93673	243-309	37	0	0.00
Puerto Inca	-9.36759	-75.00175	202-229	23	0	0.00
San Pedro Market, Cusco*	-13.52384	-71.97128	3363	2	1	0.50
Satipo	-11.27665	-74.64673	719	23	0	0.00
Shima Venzo	-11.11832	-74.22433	394-474	37	0	0.00
Tambopata Research Center	-13.13333	-69.60000	350	61	0	0.00
Wayqecha	-13.17956	-71.60561	2623-3240	15	1	0.07

*Specimen purchased from market

Table 2. Summary of study amphibian taxa tested for *Batrachochytrium dendrobatidis (Bd)* in Peru. N=number of individuals.

Family	**N**	***Bd* Positive**	**Prevalence**	***Bd*-positive Species**
Aromobatidae	8	1	0.13	*Allobates marchesiansus*
Bufonidae	166	0	0.00	
Caeciliidae	1	0	0.00	
Centrolenidae	4	0	0.00	
Ceratophryidae	3	1	0.33	*Telmatobius c.f. marmoratus**
Dendrobatidae	242	1	0.01	*Hyloxalus shuar*
Hemiphractidae	16	1	0.06	*Gastrotheca excubitor*
Hylidae	212	4	0.02	*Osteocephalus buckleyi, Hyloscirtus c.f. phyllognatus, Scinax garbei, Hypsiboas melanopleura*
Leiuperidae	12	1	0.08	*Engystomops petersi*
Leptodactylidae	139	2	0.01	*Leptodactylus c.f. andreae, Leptodactylus petersii*
Microhylidae	21	0	0.00	
Plethodontidae	4	0	0.00	
Ranidae	1	0	0.00	
Strabomantidae	154	0	0.00	

*Specimen purchased from market

Table 3. Studies of *Batrachochytrium dendrobatidis (Bd)* in Peru conducted to date.

Locality	Department	Study Year(s)	Altitude (m)	N	Prevalence	Citation
Provincia de Pataz	Piura	1999	2620	3	0.67	Venegas et al. 2008
Sibinacocha watershed	Cusco	2002	4450	4	0.75	Seimon et al. 2005
Sibinacocha watershed	Cusco	2003	4422-5244	24	0.21	Seimon et al. 2007
Cainarachi valley	San Martín	2003	600	1	1.00	Lötters et al. 2005
Sibinacocha watershed	Cusco	2005	5348	2	0.50	Seimon et al. 2007
Abra Huallahualla	Cusco	2008	3100–4550	65	0.26	Catenazzi et al. 2011
Abra Malaga	Cusco	2008	3300–4050	8	0.13	Catenazzi et al. 2011
Kosnipata Valley	Cusco	2007, 2008	1250-3700	1097	0.12	Catenazzi et al. 2011
Privada Huiquilla	Amazonas	2007, 2008	2800	23	0.00	Encisco et al. 2008

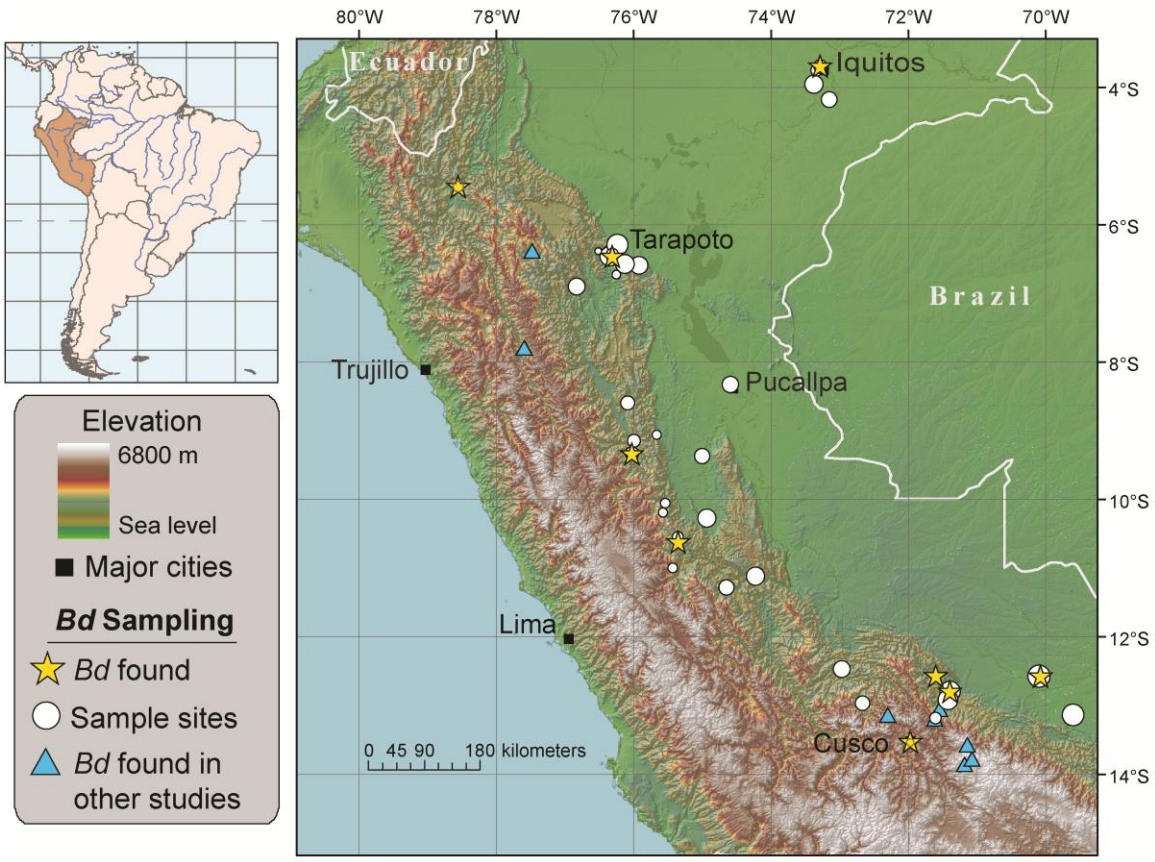

Figure 2. Study sites in Peru sampled for *Batrachochytrium dendrobatidis* (*Bd*). Circles = sample sites with circle diameter proportional to number of samples; stars= sites where *Bd* was detected, and triangles= *Bd*-positive sites reported by others (see Table 3).

Figure 3. Three species that tested positive for *Batrachochytrium dendrobatidis* (*Bd*) in this study: A) *Hyloscirtus c.f. phyllognatus*, B) *Telmatobius c.f. marmoratus*, and C) *Gastrotheca excubitor*.

CHAPTER 3: COMPARISON OF TECHNIQUES FOR FIELD SAMPLING OF *BATRACHOCHYTRIUM DENDROBATIDIS* USING RAYON SWABS

Abstract

The majority of methods papers for *Batrachochytrium dendrobatidis* (*Bd*) to date have focused on techniques for sampling *Bd* in developed countries. In order to facilitate the increased sampling for *Bd* in other areas (particularly biodiversity hotspots), it is imperative to improve the feasibility and reliability of testing for *Bd* in developing countries. In this study we compare the use of 95% ethanol, DMSO-NaCl solution, and dry swabs for sample preservation, DNeasy vs. PrepMan Ultra for DNA extraction, and end-point PCR vs. quantitative (qPCR) for *Bd* detection. Our results show that the most advantageous techniques for a *Bd*-field study are swab preservation in 95% ethanol, DNA extraction with DNeasy, and the end-point PCR disease assay. Here we outline the protocols, show the results of our methods comparisons, and discuss the pros and cons of each method.

Introduction

The amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been implicated as the worst infectious disease ever detected in vertebrates (Gascon et al. 2007). This pandemic has already been implicated in the extinction of 159 species of amphibians (Vié et al. 2009) and as it continues to spread is likely to be the cause of many more extinctions.

The majority of methods papers to date have focused on techniques for sampling *Bd* in developed countries, where most investigations have taken place. In order to facilitate increased sampling for *Bd* in developing regions (particularly biodiversity hotspots, i.e., the Amazon basin, Central Africa, South East Asia), it is imperative to improve the feasibility and reliability of testing for *Bd* in these areas. Many of the major constraints of working in developing countries involve refrigeration of samples, exportation, and obtaining supplies. Here we address these issues in our comparison of methods for sample preservation, DNA extraction, and *Bd* detection.

The preservation of amphibian skin swabs for *Bd* detection has been discussed by others (e.g., Hyatt et al. 2007, Van Sluys et al. 2008), but has not been tested for long durations (i.e., beyond 14 or 7 days respectively) under field temperatures. Here we test three DNA preservation techniques: dry preservation, 95% ethanol, and DMSO-NaCl solution (hereafter referred to as DMSO solution) for *Bd* samples collected with rayon swabs.

The majority of *Bd* investigations to date have used PrepMan Ultra (ABI), following the methods first outlined in Boyle et al. (2004) for DNA extraction. Using this method has several drawbacks. First, a tissue homogenizer is required for lysing cells. A tissue homogenizer is not a standard piece of equipment in most laboratories and can add substantial cost to a project. Additionally, the procedure calls for the use of zirconium/silica beads to aid in homogenization

and special tubes that can withstand the stress of homogenization; this further increases preparation time and costs. Lastly, although this method works fairly well with pure cell cultures, problems may occur when using PrepMan Ultra to extract DNA from field samples because this technique does not have washing steps to remove PCR inhibitors such as soil and leaf litter contaminants.

Hyatt et al. (2007) noted that PCR inhibitors are one of the leading causes of failure to detect *Bd*-positive individuals. For this reason, they developed a method for testing for the presence of inhibitors. This adds an extra step to the PCR assay, and considerably increases the cost (Internal positive control agent is $230 for 200 reactions, ABI). The DNeasy kit (Qiagen) has multiple advantages in comparison with the PrepMan Ultra technique. All the supplies for performing the DNeasy procedure come complete in a user-friendly kit; no additional laboratory equipment beyond the standard microfuge, water bath and vortex are required. This technique also utilizes multiple washing steps and a DNA column, thus, the end product is colorless and much less likely to contain PCR inhibitors (Qiagen, July 2006). Here we compare the effectiveness, convenience, and costs of PrepMan Ultra and DNeasy.

The recommended procedure for *Bd* detection is currently quantitative (qPCR) using the Taqman probe (Kriger et al. 2006, Hyatt et al. 2007, but see Garland et al. 2011). However, due to some drawbacks of this method (e.g., high start-up costs, frequent background amplification), we attempted to develop an end-point PCR method with sensitivity and reliability comparable to the widely used qPCR. Because there are already several papers published that discuss the analytical sensitivity of qPCR (e.g., Boyle et al. 2004, Kriger et al. 2006), we will not evaluate this method here. Instead, we will compare the results of our end-point PCR to those already published for qPCR. Multiple papers have been published outlining various components and

techniques of the *Bd* assay (e.g., Hyatt et al. 2007). Due to the important conservation implications of detecting this organism in a population, it is crucial that researchers use standardized, consistent methods. Consistency is critical to minimize the occurrence of false negatives during sampling efforts. Here we compare various widely-used methods for 1) DNA extraction from swabs, 2) swab preservation, and 3) detection of the disease organism. For each technique we will compare procedure times, cost, sensitivity, likelihood of error, and qualitative and quantitative output. Our hope is that providing an easy, cost effective method will promote research by smaller laboratories and provide a means for researchers unfamiliar with molecular techniques to perform in-house *Bd* assays.

Materials and Methods

Bd culturing, cell counts. The *Bd* strain used in these comparisons was originally obtained from Joyce Longcore (Jel 423) in December of 2009. Cultures were maintained on Petri plates at 25°C using tryptone agar (5 g tryptone, 12 g agar, 500 ml distilled water) and using aseptic techniques (Longcore et al. 1999). To collect zoospores, plates were flooded with sterile distilled water and incubated at room temperature for 20 minutes. Excess liquid was collected from plates, and resulting zoospore solution was mixed 1:1 with Trypan Blue (Invitrogen) to improve the ease of counting cells. Cell density then was estimated using a hemocytometer and a serial dilution was performed to dilute cell concentrations to 100, 10 and 1 zoospores/ml as described in Boyle et al. (2004). Differing cell concentrations were utilized for each of our method comparisons so that the sensitivity of each method at detecting low level DNA concentrations could be examined (as would be expected from field samples collected during earlier stages on infection).

Swab preservation. We compared three methods of swab preservation (dry, DMSO, and 95% ethanol) using two different *Bd* sources (from culture and infected frogs) for their ability to preserve DNA. Before swab collection, microvials were prepared by adding either 1000 μL of DMSO solution (DMSO solution prepared using the protocol described in Dawson et al. 1998) or 95% ethanol. Microvials for the dry swabs treatment did not require preparation. *Bd* swabs from culture then were prepared by immersing sterile swabs (MW113, Medical Wire and Equipment) into tubes with the various cell concentration standards set up previously. Each swab was immersed in the solution for five seconds and cell absorption was maximized by gently twisting the swab between the thumb and forefinger. Four swabs were collected from each of three different live, wild-caught frogs known to be infected with *Bd* (*Hyla versicolor* complex).

Each frog was swabbed systematically (i.e., 10 times on the dorsum, 10 times on the venter, five times on each side, and five times on each foot, Kriger et al. 2006b).

For our *Bd* in culture swab preservation comparison, a 3x3x2 full factorial design was used to ensure all possible combinations of treatments among experimental groups. Groups consisted of: 95% ethanol, dry or DMSO solution→ 5 or 30°C → 1, 10, or 100 zoospores/ml. Five replicates were constructed for each experimental unit resulting in a total of 90 replicates along with six negative controls (swabs inoculated with sterile water to ensure that cross contamination did not occur between replicates). All swab replicates were prepared at the same time using the same stock zoospore concentrates. Swabs were incubated at the desired temperature for 90 days.

Swabs from individual live frogs were divided into treatment groups of: 1) 95% ethanol, 6 days; 2) 95% ethanol, 68 days; 3) Dry, 6 days; and 4) Dry, 68 days. Ethanol treatment swabs were preserved in 95% ethanol immediately after swabbing until DNA extraction (after six or 68 days). Dry treatment swabs were placed in microvials immediately after swabbing with no preservative until DNA extraction (after 6 or 68 days). All swabs were preserved in an incubator at 30°C until DNA extraction.

At the end of the incubation period, DNA was immediately extracted from swabs using the DNeasy methods outlined below. Replicates then were amplified using independent end-point PCRs (see PCR section for methods), and the resulting products were compared by examining electrophoresis gels for the presence/absence of a 300 bp band.

Before DNA extraction, swabs preserved with 95% ethanol or DMSO were spun down for three minutes at 13,000 rpm in a microfuge to pellet excess material. The majority of the

supernatant fluid then was gently removed with a pipette. After this, the ethanol tubes were placed in an incubator overnight (with caps off) to evaporate off the remaining ethanol. DMSO tubes were first incubated overnight with 1000 µl of DNA free water to remove excess salts, and after removal of excess water with a pipette, they were incubated a second night (with caps off) for desiccation. Dry samples did not require preparation for DNA extraction. To increase sample yield, all DNA extractions were performed in the original microvial (from the sample preservation procedure) with the desiccated swab.

DNA extraction techniques. A DNA extraction comparison was performed using *Bd* swabs prepared from serially diluted zoospores as outlined above. Treatment groups consisted of: DNeasy or PrepMan Ultra→ 1, 10 or 100 zoospores. Five replicates were constructed for each experimental unit resulting in a total of 30 replicates. Prior to DNA extraction, swabs were incubated dry at 5°C for 24 hours.

The PrepMan Ultra procedure we tested is discussed in detail in Hyatt et al. (2007). Briefly, swabs were placed in microvials containing 30-40 mg of zirconium/silica beads (0.5 mm diameter, BioSpec Products) after which 50 µl of PrepMan Ultra was added to each tube. Tubes then were placed in the tissue homogenizer (Mini Beadbeater, BioSpec Products) for 45 seconds followed by centrifugation (30 seconds at 13,000 rpm). These homogenization and centrifugation steps were repeated once. Lastly, tubes were placed in a hot block at (10 minutes at 100°C) and resulting supernatant fluid (approximately 20 µl) was transferred into new vials and immediately frozen. Because undiluted preparations of PrepMan Ultra have been shown to be inhibitory (Boyle et al. 2004), we diluted samples 10^{-1} before setting up PCR.

We used a modified version of the DNeasy procedure for extraction of DNA from animal tissues (Qiagen, July 2006). Swabs were placed in individual tubes with 180 µl of 1:1 Buffer ATL and 20 µl of Proteinase K, vortexed and then incubated at (3 hours at 56°C). After incubation, 400 µl of Buffer AL: 95% ethanol was added to each tube, vials were then vortexed, and the resulting supernatant fluid was added to DNA spin columns (Qiagen). Spin columns were centrifuged (1 minute at 8,000 rpm) and resulting flow through discarded. Next, 500 µl of Buffer AW1 was added followed by centrifugation (1 minute at 8,000 rpm), the resulting flow through was discarded. After this, 500 µl of Buffer AW2 was added and centrifugation was performed (3 minutes at 13,000 rpm). Spin columns then were moved to new microcentrifuge tubes and incubated with 200 µl of Buffer AE for 1 minute at room temperature followed by centrifugation (1 minute at 8,000 rpm). This process was repeated once, and then tubes were immediately frozen. Replicates from all treatments were amplified using end-point PCR (see PCR section for methods) and the concentration of the resulting products was compared using gel electrophoresis.

Bd assay. An end-point PCR assay was optimized for detection of low levels of *Bd* DNA using the primer pair (*Bd*1a: 5'-CAGTGTGCCATATGTCACG-3', *Bd*2a: 5'-CATGGTTCATATCTGTCCAG-3') developed by Annis et al. (2004). Reaction recipe was as follows: 1.25 µl of DNA Gold Buffer (10x, ABI), 1.0 µl of $MgCl_2$ (16.7 µM), 2.4 µl of GeneAmp dNTP mix with dTTP (1000 µM, ABI), 0.5 µl of forward and reverse primers (5 µM, Invitrogen), 0.06 µl of AmpliTaq Gold with GeneAmp (5 units/µl, ABI), 4.29 µl of DNA grade water, 2.0 µl of sample DNA extract for a total reaction volume of 12 µl. Reactions were loaded into a 96-well plate to increase efficiency and covered with aluminum film (ABI) and placed in a PTC-200 thermal cycler (Gene Tools).

We developed the following thermal cycler program for use with our PCR master mix: 1) 5 minutes at 95°C, 2) 45 seconds at 93°C, 3) 45 seconds at 60°C, 4) 1 minute at 72°C, 5) repeat steps # 2-4 for 44 more times, and 6) 10 minutes at 72°C. The resulting product (8 µl) was mixed with 1 µl of loading dye (6X; Apex) loaded into a gel (8 ml of 1x TBE Buffer, 1.2 g of agarose; Apex). Additionally, one well per row was loaded with 100 bp DNA ladder (Promega), so that the size of the PCR amplicons could be estimated. Gels were immersed in 1x TBE Buffer and 120 volts of current then were passed through for approximately 40 minutes, after which gels were examined and photographed using a UV-viewing apparatus. Wells containing a 300 bp band were deemed positive. The analytical sensitivity (i.e., lowest detectable concentration) of this method was compared to that of published results for qPCR (0.1 zoospores, Boyle et al. 2004) using a *Bd* molecular standard obtained from Pisces Molecular LLC (Boulder, CO).

Sequencing. In addition to testing the effectiveness of PrepMan Ultra and DNeasy at yielding sufficient quantities of DNA for a PCR-based disease assay, we also were interested in whether or not extraction technique influences the "quality" of the DNA for sequencing. This was investigated using a subset of confirmed positive samples of differing zoospore concentrations from the extraction comparison and five primers that have been previously shown to amplify Single Nucleotide Polymorphism (SNP) containing loci (Morehouse et al. 2007, Fisher et al. 2009): C5(*Bd*C5F:5'TAATAGCGCCGACCGAACTA-3', *Bd*C5R: 5'-ATGCCAAACCATGAGCAAAT-3'), C18(*Bd*C18F: 5'-GCGAATACGACTGCAAATGA-3', *Bd*C18R: 5'-TGAGCTCTAGCCGACATTGA-3'), C24(*Bd*C24F: 5'-GACAATGTGCTCACGGCTTA-3', *Bd*C24R: 5'-CTCTCCAAGGCTGAATCTGG-3'), 839 (8392X2F: 5'-CATCGGGTTTGTCATTGCCTGC-3', 8392X2R: 5'-TATGGCATGTGGTCTACTCTGTCC-3'), and 870 (8702X2F: 5'-

GGATCTGCCAGTTTCGATCTACTCG-3', 8702X2R: 5'-GAATATGGCATGGGAGAAGTAGCC-3'). PCR's for the SNP primers were set up using the same parameters as outlined above in the *Bd* end-point PCR. PCR products were purified for sequencing by performing an ExoSAP-IT clean-up reaction (USB), and sequenced using a 3130 Genetic Analyzer (ABI) and BigDyeTerminator v3.1 Cycle Sequencing Kit (ABI). Resulting sequences were aligned for product verification using Sequencher (v. 4.1.4) and Mesquite (v. 2.74) and alignments were examined qualitatively for accuracy.

Analysis

Scoring electrophoresis gels. In order to compare the effectiveness of each of the procedures tested, electrophoresis gels were scored for the presence/absence of bands of the appropriate length for the primer pair used in the reaction (e.g., the 300 bp band when using the *Bd*1A/*Bd*2A primers). Replicates were termed "experimental positive" if the appropriate band was detected on the electrophoresis gel and "true positive" if the replicate was known to contain *Bd* DNA template at the beginning of the experiment. Presence/absence data from each experiment was then used to construct a Chi-square 2x2 contingency table from which p-values were calculated in order to test the null hypothesis that results were independent of treatment.

Results

Swab preservation comparison. There was no difference in the likelihood of detecting *Bd* among swab preservation techniques when using swabs from *Bd* grown in culture (Table 4, Figure 4). When performing the same comparison using swabs from live frogs, however, the 95% ethanol technique worked marginally better (6 days: x^2=6, df=1, p=0.0143, 68 days: x^2=2.4, df=1, p=0.1213, Table 5). Preparation and processing time was greatest in the DMSO and 95% ethanol preservation techniques and lowest in the dry preservation method (Table 6).

DNA extraction techniques. The detection rate of *Bd* was significantly greater in three out of six of the primer pairs tested when using DNeasy as the extraction agent (870: x^2=5.3333, df=1, p=0.0209, C24: x^2=5.3333, df=1, p=0.0209, C5: x^2=8.5714, df=1, p=0.0034, Table 7, Figure 5). DNA sequences of PCR products did not differ qualitatively among treatments: the height, clarity and distinguishability of peaks in the electropherograms derived from the respective treatments were similar (results not shown). Efficiency also was greater in the DNeasy treatment as estimated by the band intensity, Figure 5). Costs for each procedure were fairly similar, especially if the additional costs of purchasing a homogenizer and internal positive controls are factored in for the PrepMan Ultra procedure (DNeasy: $2.33/rnx vs. PrepMan Ultra: $1.70/rnx, Table 8). With regard to procedure duration, the DNeasy procedure takes longer (17 vs. 11 minutes), especially if the incubation period is factored in (197 vs. 21 minutes, Table 8).

Bd assay. Our end-point PCR was capable of reliably detecting *Bd* DNA molecule quantities as low as 0.275 molecules (or approximately 0.0016 zoospores, Table 9, Figure 6).The cost of performing the *Bd*-PCR assay, excluding equipment, was approximately $0.42 per sample; in contrast, the cost per sample for the Taqman qPCR assay was approximately $1.51 (Table 9). An

analysis of time per procedure showed that the Taqman qPCR assay takes considerably less time than the end-point PCR assay (88 vs. 265 minutes respectively, Table 9).

Discussion

Swab preservation comparison. Although the swab preservation techniques we tested worked similarly well across treatments (Table 4, Figure 4), there are some caveats to consider when choosing a field preservation method. First, the DMSO and ethanol methods have longer preparation and processing times (Table 6), which may be problematic when working with a large volume of samples. Second, some airlines and shipping companies have restrictions for shipping or exporting flammable or hazardous chemicals. Third, liquids can add a considerable amount of weight to baggage, especially if the preservatives cannot be purchased in study area and must be transported in bulk. Lastly, although the dry preservation method may seem the most attractive due to decreased preparation and processing time and ease of shipping, potential problems may arise with this method when storing swabs from live frogs for long periods of time at optimal temperatures for microbial growth as these organisms may interfere with detection of *Bd*. Overall, none of the preservation methods that we tested stood out above the rest as being the best for general use, so we recommend choosing the technique that works best based upon one's study system.

DNA extraction. The DNeasy method outperformed PrepMan Ultra in efficiency (Figure 5). This contrasts with the results of Boyle et al. (2004) which reported that PrepMan Ultra was marginally more efficient than DNeasy. DNeasy also outperformed PrepMan Ultra in sequencibility of extracts (results not shown) and in consistency (as determined by the number of true positives; Figure 5, Table 7). This was especially obvious in the replicates with lower initial zoospore quantity (i.e., 1 and 10 zoospores/ml). Because many field-collected samples may have low quantities of *Bd* (e.g., earlier infection stages, resistant individuals), using PrepMan Ultra as a DNA extraction agent can potentially lead to failure to detect the presence of the disease in *Bd*-

positive individuals. PrepMan Ultra has two other drawbacks. This method produces a lesser product volume (i.e., 20 µl vs. 400 µl with DNeasy), which can be problematic if one plans to perform multiple reactions. PrepMan Ultra also may be less capable of removing PCR inhibitors (as indicated by yellowish coloration after extraction of field samples, data not shown). PCR inhibitors are often present in field samples and will therefore increase the risk of obtaining false negatives. The drawbacks to the DNeasy method are the higher product costs and greater processing time (Table 8); however, these minor issues are more than made up for by the lower likelihood of getting false negative results.

End-point PCR assay. Using the Taqman qPCR assay, Kirshstein et al. (2007) found that *Bd* zoospores contain 169 copies of the *Bd* ribosomal RNA gene ITS-1 region. This falls within the range of 60 to 220 found for other fungi (Simon et al. 2005). Using the copy number estimate of Kirshstein et al. (2007) of 169 DNA copies per zoospore we estimate that our endpoint PCR assay (Figure 6) is similar in sensitivity if not more sensitive than the qPCR Taqman assay (0.275 DNA molecules or 0.0016 zoospores vs. 16.9 DNA molecules or 0.1 zoospores reported by Boyle et al. 2004 and Kriger et al. 2006). Unfortunately, it is not possible to make a direct comparison of assay sensitivity because our *Bd* standard was prepared and quantified differently than that used by Boyle et al. (2004). Since, the Boyle *Bd* standard is no longer being distributed, the standard used in this sensitivity test was a plasmid molecular standard purchased from Pisces Molecular. This standard is prepared by inserting the *Bd* ribosomal RNA gene ITS-1 region into a plasmid and then quantifying plasmids (previously replicated in *E. coli*) using a spectrophotometer. Because preparations of standards using this method are less variable than standard preparations that count cells with a hemocytometer (e.g., the Boyle standard, Ludwig and Schleifer 2000, Haque et al. 2003), we are reasonably confident of our results (assuming

that the number of DNA template molecules per zoospore reported by Kirshstein et al. (2007) is accurate).

Other major factors to consider when choosing between end-point PCR and qPCR are specificity, costs, procedure duration, and project goals. One of the major benefits of end-point PCR is that it has greater specificity (ability to correctly distinguish between positive and negative samples) than qPCR due to differences in interpretation of results. End-point PCR results are examined by performing gel electrophoresis and looking at the resulting bands. When performing the *Bd* assay using the primers developed by Annis et al. (2004), the electrophoresis procedure produces a 300 bp band in *Bd*-positive individuals which can easily be distinguished from primer dimers (bands <50 bp). In contrast, qPCR results are analyzed using a computer readout, which displays information conveying strength of fluorescence or product accumulation vs. cycle number. The main problem with using qPCR for presence/absence studies is that positive results can sometimes be ambiguous. This is because qPCR does not distinguish DNA fragments by size, but rather by fluorescence. Since there is always some degree of nonspecific binding of the fluorescent dyes, the prevalence of background noise can make it difficult to definitively distinguish between *Bd*-positive or negative individuals (especially if the infection load is small). Another benefit of end-point PCR is that initial equipment and reagent costs are considerably lower than the Taqman qPCR assay (Table 9).

Although qPCR does have some drawbacks, one of the major benefits to this technique is that it allows the researcher to estimate the quantity of DNA in the original sample. This estimate is performed by comparing the reaction Ct value against a standard curve made from samples of known concentrations. Estimation of DNA quantity often is used to approximate disease severity in chytridiomycosis studies (e.g., Kriger et al. 2007, Briggs et al. 2010). However, as attractive

as this method may seem for estimating host infection load, it has been criticized by others for methodological errors (e.g., reproducibility among samples and between labs, Wong and Medrano 2005). When performing amplification at a logarithmic scale, such problems can lead to considerable variation among samples and minor differences such as host size, swabbing method, pipetting techniques, and reagent age or batch number can have a dramatic effect on quantification (Freeman et al. 1999, Wong and Medrano 2005). Another benefit of using qPCR is that the procedure is considerably less time-consuming; fewer cycles are required for target detection and gel electrophoresis is unnecessary. The faster throughput time of qPCR may make it an advantageous technique for some studies, especially when time is extremely limited or for large-scale sampling efforts.

Overall, although qPCR has some advantages relative to end-point PCR (e.g., quantification and speed), we believe that end-point PCR is the better method for *Bd* disease assays. End-point PCR clearly distinguishes between positive and negative samples, there is a lower risk of minor variations in protocol leading to large differences in results, and it is considerably less expensive.

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Tables and Figures

Table 4. Ratio of experimental positives to true positives of rayon swabs from *Bd* culture detected using the end-point PCR assay for each preservation method. Zs=zoospores.

	Temperature		**Zoospore concentration**		
Preservative	**5°C**	**30°C**	**100 zs/ml**	**10 zs/ml**	**1 zs/ml**
95% Ethanol	15/15	14/14	5/5	5/5	4/4
Dry	15/15	15/15	5/5	5/5	5/5
DMSO	14/15	13/15	4/5	5/5	5/5

Table 5. Ratio of experimental positives to true positives of rayon swabs from *Bd* infected frogs detected using the end-point PCR assay for each preservation method.

Preservative	**Preservation time**	
	6 days	**68 days**
95% ethanol	6/6	6/6
Dry	2/6	4/6
P-Value	0.0143	0.1213

Table 6. Comparison of DNA preservation techniques.

Preservative	Cost/sample	Extra Preparation Required	Extra Processing Time	Potential Issues w/ Field Work
95% ethanol	$0.05	yes	24 hours	More weight, shipping restrictions due to flammability
Dry	$0.00	no	none	Microbial growth interference w/DNA yield
DMSO	$0.10	yes	48 hours	More weight, shipping restrictions due liquid hazard level

Table 7. Ratio of experimental positives to true positives detected using the end-point PCR assay for each the two extraction methods and six different primers tested.

Extraction Method	**Primers**					
	Bd	**870**	**C24**	**C5**	**C18**	**839**
DNeasy	14/15	5/6	5/6	6/6	4/6	4/6
PrepMan Ultra	12/15	1/6	1/6	1/6	2/6	2/6
P-Value	0.6215	0.0209	0.0209	0.0034	0.2482	0.2482

Table 8. Comparison of DNA extraction techniques.

Extraction Method	Cost/sample	Time/sample	Yield	Sequencible Products	Extra Equipment
DNeasy	$2.33	17 min*	400 µl	yes	no
PrepMan Ultra	$1.70	11 min**	20 µl	some	yes

*Plus 3 hours for incubation, **Plus 10 minutes incubation

Table 9. Comparison *of Batrachochytrium dendrobatidis* (*Bd*) PCR assays. Zs=zoospores.

Extraction Method	Cost/sample	Thermal Cycler Cost	Time/sample	Sensitivity	False Positives	Sample Quantification
End Point	$0.42	$3,800	265 min*	0.1 zs	no	no
qPCR	$1.51	$38,500	88 min*	0.275 molecules	yes	yes

*Includes time in thermal cycler

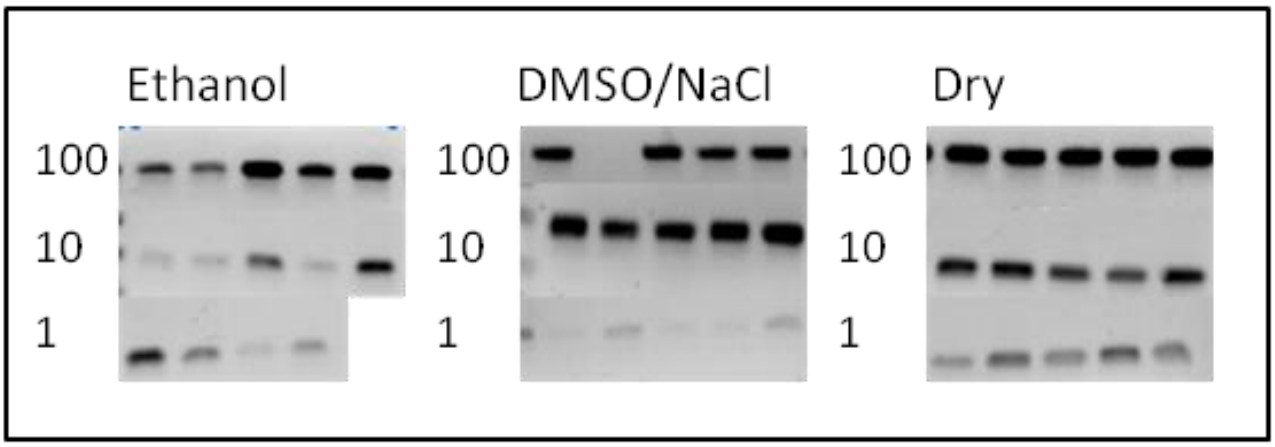

Figure 4. Electrophoresis gel images from DNA preservation methods comparison. Swabs were stored at 30°C for 90 days before extraction.

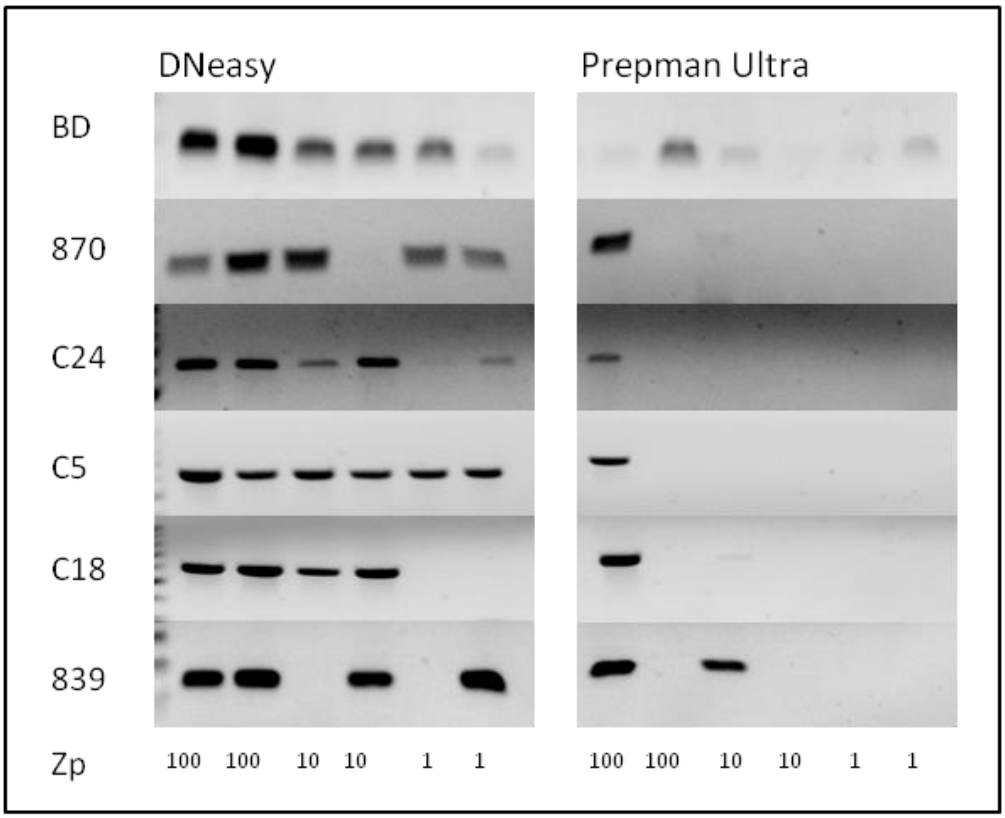

Figure 5. Representative gels for DNA extraction methods comparison. Zp=zoospore count/ml.

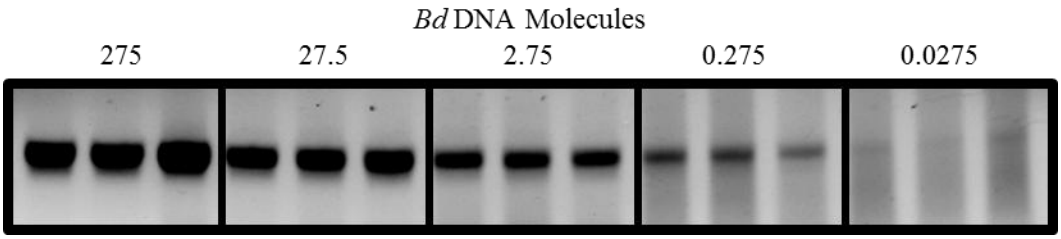

Figure 6. Electrophoresis gel image from end-point PCR sensitivity assay.

CHAPTER 4: TECHNIQUES FOR MINIMIZING THE EFFECT OF PCR INHIBITORS IN THE CHYTRIDIOMYCOSIS ASSAY

Abstract

Since the discovery of *Batrachochytrium dendrobatidis* (*Bd*) in 1998, several methods have been utilized for detection; among these PCR from skin swabs is accepted as the best method due to its high sensitivity, non-invasiveness and ease of use. However, PCR is a chemical reaction that is dependent upon the presence of non-degraded DNA template and reaction components that do not inhibit the process. These ingredients are crucial in order for the amplification reaction to proceed and accurate detection of the pathogen to occur. Here, we report on a comparison of four techniques that can be utilized for DNA extraction and of four techniques for analyzing *Bd* samples post-DNA extraction which minimize the inhibitory effects of humic acid on PCR. In order to dramatically improve the reliability of the *Bd* PCR assay, we recommend the use of the DNeasy kit (Qiagen) for DNA extraction and an end-point PCR assay with Amplitaq Gold Taq polymerase (ABI) and bovine serum albumin (BSA) due their effectiveness at removing humic acids, low cost, and relative ease of use.

Introduction

Chytridiomycosis is an emerging infectious disease of amphibians caused by the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter *Bd*). This disease has contributed to major declines and extinctions of amphibians worldwide (Fisher et al. 2009). Since its discovery and subsequent description, detection techniques for chytridiomycosis have improved considerably in reliability and sensitivity. However, as we will demonstrate, there are still many issues with the *Bd* assay that need to be resolved before it will be up to par with the quality of standard assays for most diseases.

Despite the high sensitivity and relative ease of use of the PCR method for *Bd* detection, the reliability of this procedure is often plagued by a high probability of false negatives (e.g., up to 72.5% of reactions in one study, Garland et al. 2010). This can have devastating consequences, especially given that disease management decisions may be influenced by this type of data. One of the main contributors to PCR failure is reaction inhibition. Such inhibition is especially problematic in field studies where contamination by exogenous substances (e.g., soil and dust) is common, and where samples frequently vary in chemical characteristics (Wilson 1997). Humic acids are cited as the main contributor to PCR inhibition in environmental samples (Wilson 1997), although other compounds such as fulvic acids, metals and polysaccharides can also have inhibitory effects (Tsai and Olson 1992). Humic acids are poly-phenolic compounds produced during the degradation of organic materials. These compounds are known to interfere with the binding of Taq polymerase (Wilson 1997), and have been shown to completely inhibit PCR when present in quantities of as low as 1ng per 50µl reaction (Kermekchiev et al. 2009). Unfortunately, due to their physico-chemical similarities to DNA (i.e., high molecular weight and negative charge), these compounds react in the same way as DNA molecules with the

reagents used in most extraction procedures (e.g., DNeasy, Qiagen), ultimately leading to their co-purification along with the target DNA (Tsai and Olson 1992).

Such problems are well understood in soil and fecal studies (e.g., Wilson 1997), but are commonly overlooked in other types disease surveys. In the literature on chytridiomycosis detection, PCR inhibition is rarely mentioned (but see Garland et al. 2010). In fact, the most cited method paper on the *Bd* assay (Boyle et al. 2004, 224 citations) does not even mention PCR inhibition. In addition, even though a more recent paper by the same authors does mention problems with inhibition (in 25% of their samples in one experiment, Hyatt et al. 2007), the only solution that they offer for this problem is dilution of "problematic" samples, a procedure that they did not test for effectiveness. Although dilution of inhibitory samples has been shown to be effective in some cases (Tsai and Olson 1992), the effectiveness of this method is sample specific, making it too unreliable for field samples (e.g., due to their high variance in inhibitor concentration, chemical make-up, and target DNA concentration, Kermekchiev et al. 2009).

Here, we test the effectiveness of several techniques developed by researchers working with soil and fecal samples for dealing with humic acid inhibition. Our study is divided into two major sections, depending upon whether the technique deals with samples pre or post-DNA extraction. For the DNA extraction component, we tested the efficacy of four DNA extraction kits at removing inhibitors: DNeasy (Qiagen), QIAamp DNA Stool (Qiagen), PowerLyzer PowerSoil (MoBio), and PrepMan Ultra (ABI). For post-DNA extraction methods, we tested the applicability of four techniques: Amplitaq Gold (ABI), Bovine Serum Albumin (BSA, Sigma Aldrich), PowerClean DNA Clean-up Kit (MoBio), and inhibitor resistant Taq Polymerase mutants (DNA Polymerase Technologies).

Materials and Methods

Preliminary Testing

The occurrence of PCR inhibition was estimated by examining PCR results for the presence/ absence of primer dimers (<50 bp bands) using 94 amphibian skin swabs from Peruvian amphibians collected in 2010. Swabs were extracted with DNeasy following the protocol detailed below. To further verify the occurrence of PCR inhibition, a subset of three samples was serially diluted and spiked with *Bd* from culture (for visualization) and then examined for the presence/absence of a 300 bp band.

Experimental Sample Preparation

Experimental samples were prepared by adding 20 µl of a previously frozen liquid culture of *Bd* (gel 197 obtained from Joyce Longcore) or distilled water (for negative controls) along with sterile rayon swabs (MW113, Medical Wire and Equipment) to 1.7 µl microfuge tubes. For consistency, all treatments were set-up from the same *Bd* culture stock tube. A dilution series of humic acid (Sigma-Aldrich) was prepared for comparisons of the methods at initial concentrations of 10, 1, 0.1, 0.01, and 0.001 µg/µl. Humic acid solution was then added to samples before performing each technique so that the final reaction concentration was consistent across all tests (0.8 to .00008 µg/µl). For simplicity, initial concentrations of humic acid will be used hereafter when referring to results. The HA concentrations for the PowerClean comparison differ from those of the other methods because the sample starting volume had to be increased to 150 µl with water for the method to be effective. Due to this disparity, pre-PowerClean results (using Amplitaq Gold) are shown with post-PowerClean results for comparison.

DNA Extraction Solutions

DNeasy. The DNeasy procedure was performed according to the Bench Protocol for Animal Tissues outlined in the Qiagen handbook (July 2006). Several steps were slightly modified: Step 2: incubation was performed for 3 hours; Step 3: Buffer AL and 95% ethanol were premixed; and Step 7: was repeated twice. Care was taken to avoid contact of the DNA column with the flow-through, (this was especially true after Step 6, as ethanol carryover can interfere with subsequent reactions, Qiagen, July 2006).

QIAamp DNA Stool. This procedure was performed following the protocol outlined in the Qiagen handbook for the Isolation of DNA from Stool for Pathogen Detection (April 2010). Initially, two lysis temperatures (70 and 95°C) for Step 3 were tested for efficacy (results not shown) as both temperatures performed similarly lysis at 70°C was utilized thereafter.

PowerLyzer PowerSoil. The PowerLyzer PowerSoil procedure was performed according to the Experienced User Protocol in the MoBio Instruction Manual (v. 05212010). The only deviations from the manual were during the homogenization step of this procedure, in which a different apparatus was used (Mini Beadbeater, BioSpec Products), and the homogenization time was extended to 50 seconds (rather than 45) to fit the minimum time settings on the device.

PrepMan Ultra. The PrepMan Ultra procedure we tested is discussed in detail in Hyatt et al (2007). Briefly, swabs were placed in microvials containing 30-40 mg of zirconium/silica beads (0.5 mm diameter, BioSpec Products) after which 50 µl of PrepMan Ultra was added to each tube. Tubes were then placed in the tissue homogenizer (Mini Beadbeater, BioSpec Products) for 50 seconds followed by centrifugation (30 seconds at 13,000 rpm). Homogenization and centrifugation steps were then repeated once. Lastly, tubes were placed in a hot block at (10

minutes at 100°C) and resulting supernatant fluid (approximately 20 µL) was transferred into new vials and immediately frozen. Because high concentrations of PrepMan Ultra have been shown to be inhibitory (Boyle et al. 2004), we diluted samples 10^{-1} before setting up PCR.

Post-DNA Extraction (with DNeasy) Solutions

BSA. BSA solution was prepared at room temperature by the addition of lyophilized BSA powder (A4161, Sigma-Aldrich) to DNA-safe water followed by gentle mixing (without vortexing, until completely dissolved). This solution was then diluted serially to obtain a final concentration of 1000 ng/µl. The final concentration of BSA in the PCR master mix was 400 ng/µl (10 µl of BSA/25 µl PCR master mix), which was previously shown to be the optimal concentration for minimizing PCR inhibition (Kreader et al. 1996, Garland et al. 2010).

PowerClean DNA Clean-up Kit. The PowerClean procedure was performed according to the Experienced User Protocol in the MoBio Instruction Manual (v. 06222010). Starting sample volume was 150 µl.

Inhibitor resistant Taq Polymerase mutants. Several inhibitor resistant Taq polymerases (Klentaq LA, Omni Klentaq LA, OmniTaq LA, Cesium Klentaq AC LA, and CesiumTaq LA) and their paired reaction buffers developed by DNA Polymerase Technologies were tested for their efficiency at replicating *Bd* DNA in the presence of inhibitors. Testing showed that a combination of Omni Klentaq LA and PCR Enhancer-2 produced the best results with the *Bd* primers used, minimizing HA inhibition (results not shown). Therefore only Omni Klentaq LA and PCR Enhancer-2 were used for the rest of the comparisons. A detailed description of this procedure is outlined below.

Verification of Methods

The Amplitaq Gold PCR with BSA was tested for its effectiveness at minimizing the effects of PCR inhibition using Peruvian amphibian skin swabs that were collected in 2011. The results were compared to that of an Amplitaq Gold PCR using the same samples. A subset of these samples that were either *Bd*-positive, *Bd*-negative, or ambiguous were then purified with PowerClean to investigate its effectiveness at removing PCR inhibitors. The results were compared to that of Amplitaq Gold PCR with BSA using the same samples.

Analysis

*End Point PCR with Amplitaq Gold***.** An end-point PCR assay previously optimized by Kosch et al. (Chapter 3) which utilizes the PCR primers (*Bd*1a: 5'-CAGTGTGCCATATGTCACG-3', *Bd*2a: 5'-CATGGTTCATATCTGTCCAG-3') developed by Annis et al. (2004) was used to analyze the effectiveness of the techniques we tested. Briefly, the reaction recipe consisted of: 2.6 µl of DNA Gold Buffer (10x, ABI), 2.08 µl of $MgCl_2$ (16.7 µM, ABI), 5.0 µl of GeneAmp dNTP mix with dTTP (1000 µM, ABI), 1.0 µl of forward and reverse primers (5 µM, Invitrogen), 0.125 µl of AmpliTaq Gold with GeneAmp (5 units/µl, ABI), 10.45 µl of DNA grade water (Fisher), 2.75 µl of sample for a total reaction volume of 25 µl. The following thermal cycler program was utilized on a MultiGene TC9600-G machine (Labnet International): 1) 5 minutes at 95°C, 2) 45 seconds at 93°C, 3) 45 seconds at 60°C, 4) 1 minute at 72°C, 5) repeat steps # 2-4 for 44 more times, and 6) 10 minutes at 72°C. This *Bd* assay was utilized for all methods comparisons unless stated otherwise.

End Point PCR with Omni Klentaq LA. The reaction recipe and thermal cycler conditions were optimized for use with the *Bd* primers developed by Annis et al. (2004, mentioned above) from the DNA Polymerase Technologies (DPT) protocol for mutant Taq polymerases. The reaction recipe consisted of: 2.5 µL of 10x Buffer (Klentaq Mutant, DPT), 5.0 µl of GeneAmp dNTP mix with dTTP (1000 µM, ABI), 1.0 µl of forward and reverse primers (5 µM, Invitrogen), 0.25 µL of Taq polymerase (Omni Klentaq LA; DPT), 12.5 µL of PCR Enhancer Cocktail-2 (PEC-2; DPT), and 2.75 µl of sample for a total reaction volume of 25 µl. Thermal cycler conditions were as follows: 1) 10 minutes at 94°C, 2) 45 seconds at 94°C, 3) 45 seconds at 60°C, 4) 2 minutes at 68°C, 5) repeat steps # 2-4 for 44 more times, and 6) 10 minutes at 68°C.

Gel electrophoresis. PCR products were separated using gel electrophoresis which allowed us to qualitatively compare the effectiveness of each method. This procedure was performed by loading 8 µL of PCR product combined with 1 µL of loading dye (6X; Apex) to individual wells of an agarose gel (90 ml of 1x TBE Buffer, 1.35 g of agarose, Apex). Additionally, one well per row was loaded with 100 bp DNA ladder (Promega), so that the size of the PCR amplicons could be estimated. Gels are then immersed in 1x TBE Buffer and 120 volts of current are passed through for approximately 40 minutes, after which gels are examined and photographed using a UV viewing apparatus.

Ease of procedure. Ease of procedure was determined by counting the number of steps (e.g., pipetting, vortexing, incubation) for each procedure.

Calculating percent improvement and percent inhibited. In order to evaluate the effect that PCR inhibition has on PCR analysis of field samples, we calculated percent improvement and percent inhibition using gel electrophoresis results from a subset of our amphibian skin swabs collected in Peru in 2011. Percent improvement was calculated as: [(total new positives detected- total known positives)/N] x 100%. Percent inhibition was calculated as: (Total lanes without primer dimers/N) x 100%.

Results

Preliminary Investigation

The absence of 300 bp bands and primer dimers (<50 bp band) from an amphibian skin swab spiked with *Bd* and previously extracted with DNeasy indicate the PCR is being inhibited at initial sample concentrations from 1 to 1/100 (Figure 7). Furthermore, a preliminary analysis of 94 amphibian skin swabs (by scoring for the presence/absence of primer dimers) indicates that PCR inhibition is occurring in 89% of amphibian skin swabs (Table 11).

DNA Extraction Solutions

Of the four DNA extraction methods tested, QIAamp DNA Stool, and PowerLyzer PowerSoil were equally effective at removing humic acid from experimental samples (maximum capability of 1 µg/µl of HA), DNeasy was the next most effective method (maximum capability of 0.1 µg/µl of HA) and PrepMan Ultra was the least effective method (maximum capability of 0.01 µg/µl of HA, Table 10, Figure 8). Although QIAamp DNA Stool and PowerLyzer PowerSoil are better at removing humic acid than DNeasy and PrepMan Ultra, the efficiency of these two methods (as indicated by the intensity of the resulting bands during electrophoresis) is lower (Figure 8).

A cost analysis of DNA extraction techniques showed that PrepMan Ultra was the least expensive method to perform with costs varying from $0.27 for PrepMan Ultra, $2.33 for DNeasy, $3.84 for QIAamp DNA Stool and $4.36 for PowerLyzer PowerSoil (Table 10).

A comparison of the number of steps per procedure showed that PrepMan Ultra had the least number of steps at nine, followed by DNeasy with 20, QIAamp DNA Stool with 32, and PowerLyzer PowerSoil with 46 (Table 10).

Post-DNA Extraction Solutions (with DNeasy)

Several different techniques were tested for their effectiveness dealing with HA post-DNA Extraction. PowerClean is a method that removes inhibitors from extracted DNA template, BSA minimizes inhibitory effects when added to the PCR by binding to inhibitory compounds (Loomis 1974), and Omni Klentaq LA is a mutant Taq polymerase that was developed to resist HA inhibition. Of these methods, PowerClean was the most effective at dealing with high HA concentrations in samples (maximum capability of 10 µg/µl of HA, Figure 10), followed by BSA combined with Amplitaq Gold (maximum capability of 0.1 µg/µl of HA), and Omni Klentaq LA (maximum capability of 0.01 µg/µl of HA, Table 10, Figure 9). All of these methods outperformed the original *Bd* PCR assay with Amplitaq Gold which was inhibited by HA concentrations as low as 0.01 µg/µl (Table 10, Figure 9).

A cost analysis of post-DNA extraction techniques showed that BSA combined with Amplitaq Gold was the least expensive at $0.4193, followed by Omni Klentaq LA at $0.78, and PowerClean at $3.32 (Table 10). All of the methods were more expensive than original *Bd* PCR assay with Amplitaq Gold, but the addition of BSA to this reaction can be done for only a minimal cost increase of $0.005 per sample (Table 10).

The number of steps for each procedure was only analyzed for PowerClean, because the increase in the number of steps with Omni Klentaq LA or BSA compared to the original *Bd* PCR

assay with Amplitaq Gold was negligible. However, use of PowerClean does increase the effort involved in screening samples for *Bd* by adding an additional 30 steps (Table 10).

Verification of Methods

The Amplitaq Gold PCR reaction with BSA was tested for its effectiveness at minimizing the effects of PCR inhibition using 94 Peruvian amphibian skin swabs that were collected in 2011 and later extracted with DNeasy. The results of a comparison of a PCR analyses performed with and without BSA indicate that four of 94 previously *Bd*- negative samples were *Bd*-positive demonstrating a 4% improvement in detection ability when using BSA (Table 11, Figure 11). A subset of 26 of the previous field samples (three *Bd*-positive, 11 *Bd*-negative, or 12 ambiguous) were then purified with PowerClean to determine if some samples were still inhibitory. Results show that the number of *Bd*-positive samples increases from three to five after PowerClean purification demonstrating an 8% improvement in detection ability when using this method (Table 11, Figure 12).

Discussion

The results of this study and Garland et al. (2010) indicate that PCR inhibition is a common occurrence in the *Bd* assay (i.e., prevalence of 89% and 72.5% respectively). Additionally, our results demonstrate that the absence of primer dimer bands can be utilized as a preliminary indicator of PCR inhibition (Figure 7).

DNA extraction is the first line of defense against PCR inhibitors; therefore, choosing an effective method is important, especially when working with amphibian skin swabs which can be chemically complex and variable. Here, I demonstrate that QIAamp DNA Stool and PowerLyzer PowerSoil are the most effective DNA extraction methods at removing high concentrations of humic acid from samples (Figure 8). Additionally, I demonstrate that the previously suggested method for *Bd* sample extraction, PrepMan Ultra (e.g., Hyatt et al. 2007) is ineffective at removing humic acid from samples at starting concentrations as low as 0.1 µg/µL (Figure 8). A comparison of cost per sample and ease of method shows that PrepMan Ultra is the cheapest and easiest method to perform followed by DNeasy, and then QIAamp DNA Stool and PowerLyzer PowerSoil (Table 10).

Our results show that Amplitaq Gold is highly sensitive to humic acid PCR inhibition (minimum inhibitory concentrations of 0.01 µg/µl, Figure 9). Of the methods that we compared for minimizing inhibitory effects of humic acid post-DNA extraction, PowerClean was the most effective at handling high humic acid concentrations followed by BSA and Omni Klentaq LA (Table 10, Figures 9 and 10). Although PowerClean was the most effective method, it was also the most expensive and time consuming post-DNA extraction procedure (Table 10).

An applicability test performed using 94 field swabs showed that the addition of BSA to the Amplitaq Gold PCR increased the likelihood of detecting *Bd* by 4% (Table 11). A later test with a subset of the previous samples showed that the likelihood of *Bd* detection was further increased by another 4% after sample cleanup with PowerClean (Table 11). Since the samples used in these tests are field samples, it is not possible to verify their *Bd* status; however, when combined with the results of our controlled laboratory study, they further validate the applicability of this method for screening for the presence of *Bd* using field swabs containing PCR inhibitors.

The combined results of this investigation suggest the importance of considering the implications of PCR inhibitors in *Bd* studies. Furthermore, these results indicate that previous *Bd* studies that have not dealt with the potential presence of inhibitors may be drastically underestimating the prevalence of this disease by at least as much as 8% (Table 11). Such results indicate that consideration needs to be taken when planning and implementing a field study for *Bd* to minimize the likelihood of detecting false negatives from PCR inhibition. Whenever possible, researchers should avoid contamination of swabs with soil or leaf litter during collection and take special precautions (e.g., use a DNA extraction method designed for HA removal such as QIAamp DNA Stool) when handling samples that are thought to contain high concentrations of these substances. Secondly, because the use of BSA is very economical, it should be added to all *Bd* PCR assays as an added precaution against PCR inhibition. Lastly, the presence/absence of primer dimers should be determined for "negative" samples since the absence of this band indicates PCR inhibition. In such cases, PowerClean can be used to remove inhibitors before repeating the PCR assay.

The results of this study highlight the vulnerability of the PCR assay to inhibition by environmental contaminants, and may have important implications in other fields that use PCR for presence/absence studies such as dietary analysis, microbiome studies, and epidemiological surveys where such factors may be overlooked.

Conclusion

Although QIAamp DNA Stool, PowerLyzer PowerSoil are the most effective methods at removing humic acid from samples, we recommend using the DNeasy kit instead because it is more rapid and economical (Table 10) as well as efficient (Figure 8 and results of previous comparison of efficiency of PrepMan Ultra and DNeasy, Chapter 3). DNA extraction efficiency is a critical component of the *Bd* assay, and this especially true when sampling amphibians in the initial stages of infection or when sequencing is involved. Although PowerClean is very effective, we recommend that it only be used for samples that are still inhibited even after the addition of BSA, as PowerClean is a very costly and time consuming method to perform (Table 10). For general use, the *Bd* PCR assay with DNeasy, BSA and Amplitaq Gold is reliable and relatively robust to HA inhibition as well as economical and easy to perform in any standard molecular lab.

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Tables and Figures

Table 10. Comparison of techniques for minimizing the effects of humic acid (HA) for *Batrachochytrium dendrobatidis* (*Bd*) PCR assay.

			Effectiveness (HA concentration µg/µl)				
Technique	**Cost/sample**	**# Steps**	**10**	**1**	**0.1**	**0.01**	**0.001**
DNeasy[1]	$ 2.33	20	no	no	yes	yes	yes
Prepman Ultra[1]	$ 0.27*	9*	no	no	no	yes	yes
QIAamp DNA Stool [1]	$ 3.84	32	no	yes*	yes	yes	yes
PowerLyzer PowerSoil [1]	$ 4.36	46	no	yes*	yes	yes	yes
Amplitaq Gold	$ 0.41875*	n/a	no	no	no	no	yes
Amplitaq Gold and BSA	$ 0.41925	n/a	no	no	yes	yes	yes
Omni Klentaq LA	$ 0.78	n/a	no	no	no	yes	yes
PowerClean	$ 3.32	30	yes*	yes	yes	yes	yes

[1] DNA extraction methods, * best per category

Table 11. Comparison of techniques for minimizing the effects of humic acid (HA) in the *Batrachochytrium dendrobatidis* (*Bd*) PCR assay using Peruvian amphibian field swabs collected in 2011 and extracted with DNeasy.

Technique	**N**	**Primer Dimers Absent**	***B*d-positive**	**Percent Improvement**	**Percent Inhibition**
Amplitaq Gold	94	84	0	n/a	89%
Amplitaq Gold and BSA	94	2	4	4%	2%
Amplitaq Gold, BSA, and PowerClean	26	0	5	8%	0%

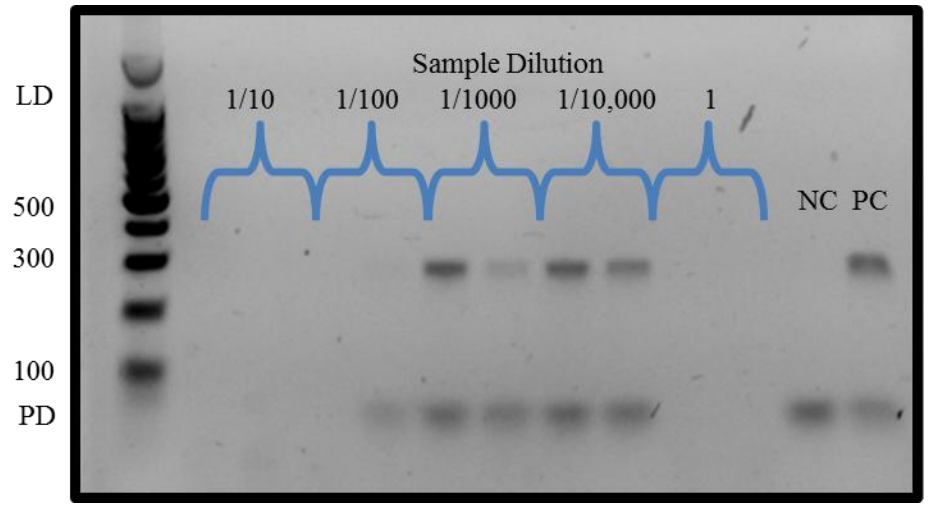

Figure 7. Serial dilution of representative inhibitory amphibian skin swab spiked with *Batrachochytrium dendrobatidis* (*Bd*) for visualization. LD=100bp DNA ladder, PD=primer dimer, NC=negative control, PC=positive control.

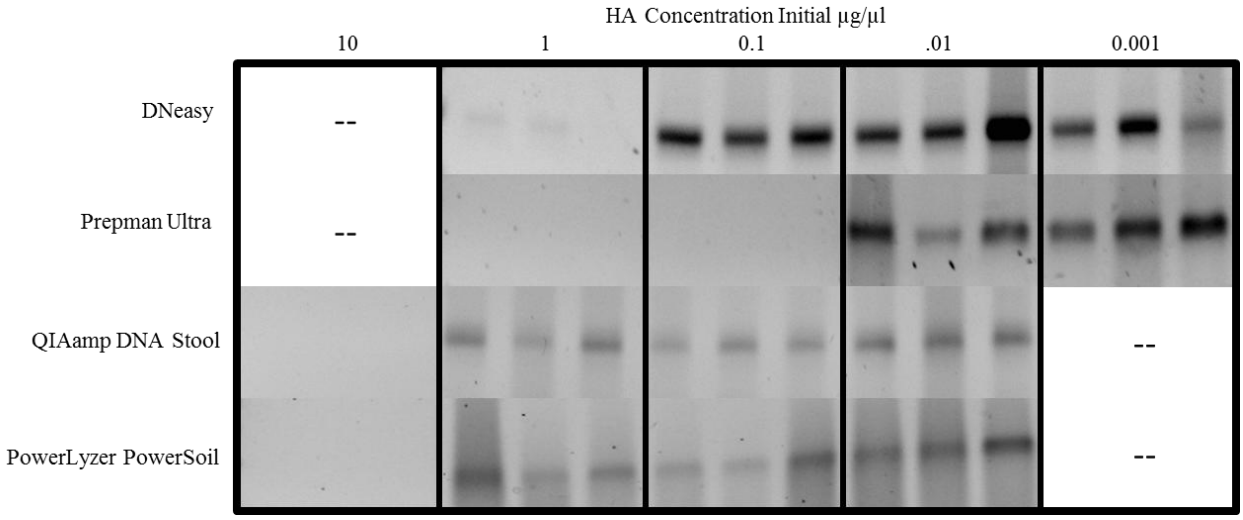

Figure 8. Comparison of the effectiveness of DNA extraction techniques at removing humic acid (HA) from *Batrachochytrium dendrobatidis* (*Bd*) samples.

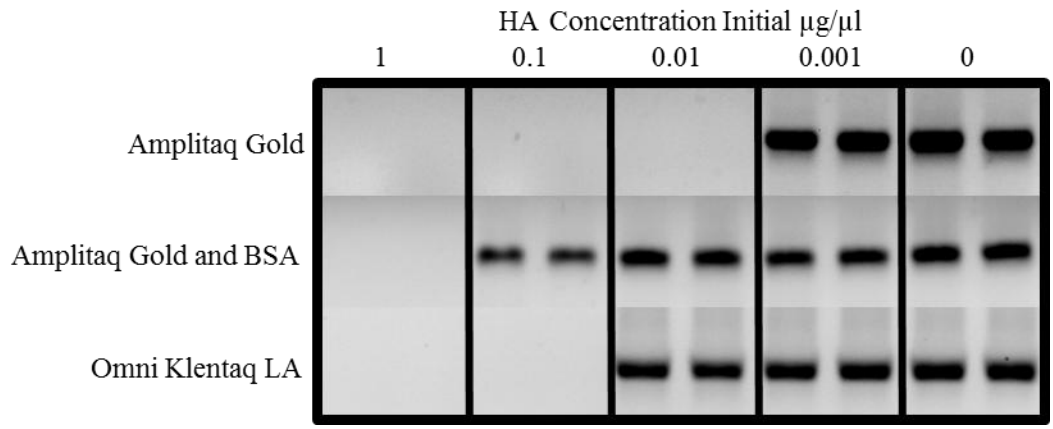

Figure 9. Comparison of techniques for minimizing the effects of humic acid (HA) from *Batrachochytrium dendrobatidis* (*Bd*) samples post-DNA extraction.

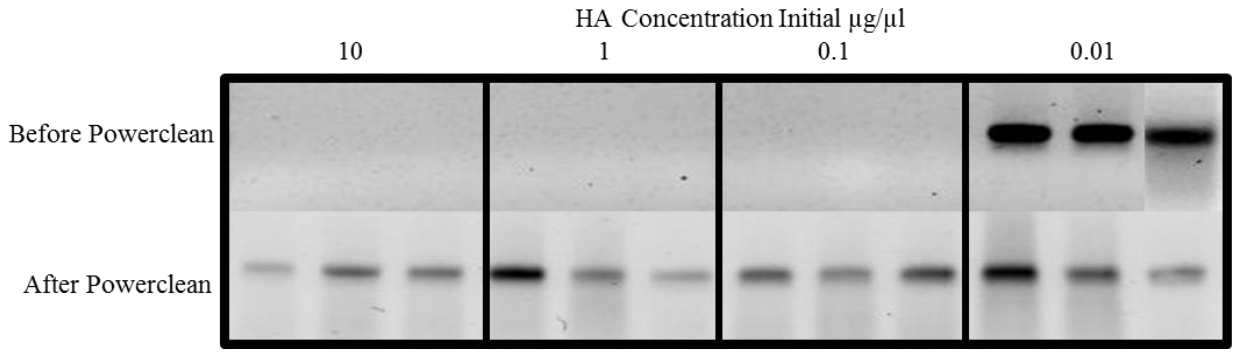

Figure 10. Comparison of the effectiveness of PowerClean at removing humic acid (HA) from *Batrachochytrium dendrobatidis* (*Bd*) samples.

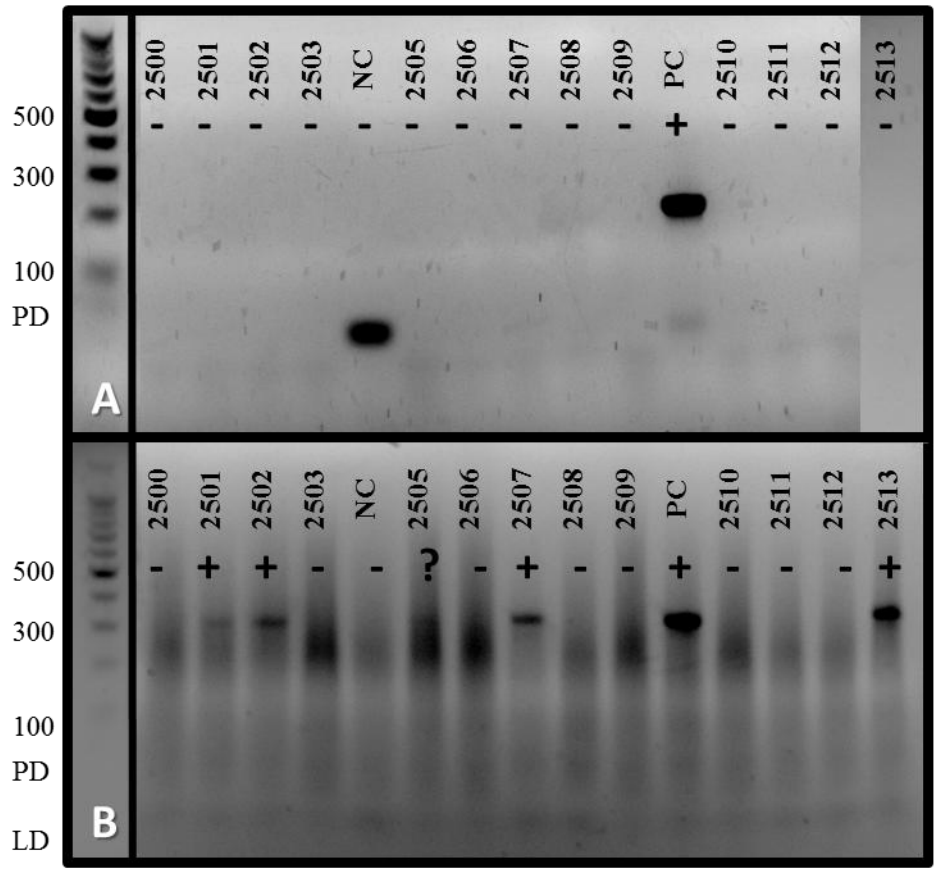

Figure 11. Representative gel images for the verification of the effectiveness of Amplitaq Gold PCR with BSA at minimizing the effects of PCR inhibitors using field samples extracted with DNeasy. A) PCR with Amplitaq Gold, B) PCR with Amplitaq Gold and BSA. LD=100bp DNA ladder, PD=primer dimer, NC=negative control, PC=positive control, **+** *Bd* positive, **-** *Bd* negative, **?** ambiguous.

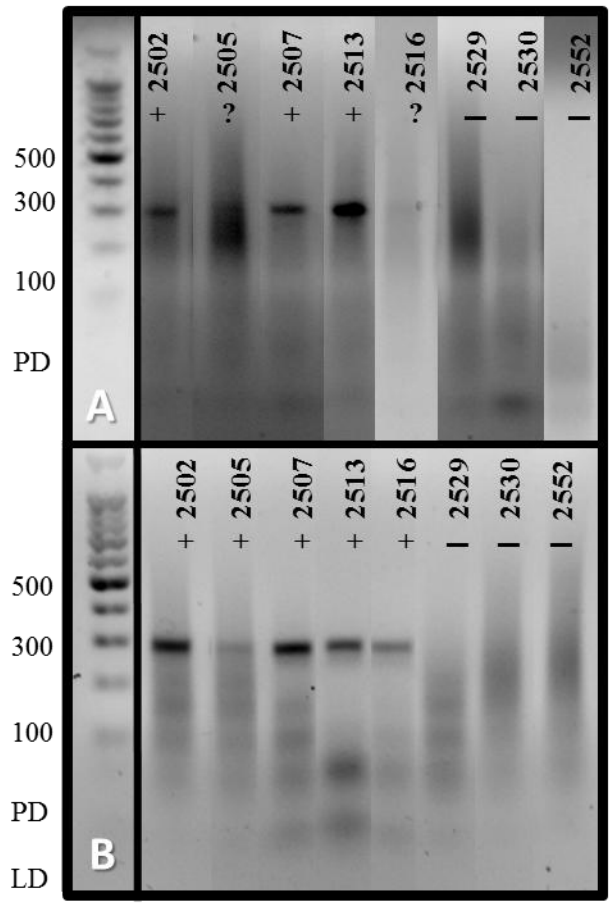

Figure 12. Representative gel images for the verification of the effectiveness of PowerClean at removing PCR inhibitors from field samples extracted with DNeasy. A) PCR with Amplitaq Gold and BSA, B) PCR with Amplitaq Gold and BSA using samples purified with Powerclean. LD=100bp DNA ladder, PD=primer dimer, NC=negative control, PC=positive control, + *Bd* positive, - *Bd* negative, **?** ambiguous.

CHAPTER 5: GENERAL OVERVIEW AND BROADER IMPACTS

Bd in Peru

In Chapter 2, I discuss the results of my sampling effort from the dry seasons of 2007 and 2008 showed that *Bd* is widely distributed throughout Peru, with among site prevalence ranging from 0 to 25%. *Bd* was detected in 11 of 983 individuals sampled, and in 9 out of 38 sites ranging in altitude from 96-3240 meters. We detected *Bd* in 11 species of anurans belonging to 14 families. The family with the highest number of *Bd*-positive individuals was Hylidae with four positive samples, all from different species. The disease also was detected in one of two *Telmatobius marmoratus* purchased at a market in Cusco where they were being sold for human consumption further supporting the idea that human transport of amphibians is facilitating the dispersal of this *Bd*. We strongly recommend further studies of *Bd* in Peru in order to better understand the ecology of this devastating disease and its effects on this biodiverse region. Although the results of this and previous studies demonstrate that *Bd* is widespread in South America (e.g., Brazil: Carnaval et al. 2006, Venezuela: Sánchez et al. 2008, Argentina: Barrionuevo and Ponssa 2008), the consequences of this disease are still relatively unknown due to the near complete absence of general population monitoring and *Bd* studies in these countries. In Peru, significant declines have been reported in multiple high-elevation species in southern Peru (Catenazzi et al. 2011), and several *Atelopus* and *Telmatobius* species are believed to already be extinct (Venegas et al. 2008, von May et al. 2008). Peru contains some of the highest amphibian diversity on record and the loss of this diversity could have severe consequences. This makes the lack of knowledge on *Bd* and population status of Peru's amphibians especially alarming.

In the discussion section of Chapter 2, I make brief mention of a theoretical model for estimating disease prevalence proposed by Cannon and Roe (1982). The authors stated that at least 30 individuals need to be swabbed per locality in order to detect at least one positive individual at a site if the disease prevalence is 10% (Cannon and Roe 1982). I reasoned that if Cannon and Roe's model holds true, I may have underestimated the actual *Bd* prevalence in localities where I was unable to collect at least 30 individuals. One potential caveat with Cannon and Roe's model is that population disease history and/or environmental conditions may influence the researcher's ability to accurately approximate disease prevalence. For example, in a population with a long history of *Bd* and/or environmental conditions non-conducive to promoting pathogenicity (e.g., a lowland population), *Bd* prevalence may be high, but mortality will be low, reflecting long term coevolution between pathogen and host and/or as a suboptimal environmental regime for the pathogen. Conversely, if sampling is performed in a population with a recent introduction of *Bd* and/or optimal environmental conditions for the pathogen (e.g., a highland population), *Bd* prevalence may be skewed. This is especially true if host population size is small and/or heterogeneously distributed, which may contribute to "pockets" of high disease prevalence distributed among regions that are relatively disease free (i.e., due to lack of disease transmission). Such information can be deceiving because it does not provide valuable insight about the disease threat of the population. In fact, high disease prevalence such as what would be detected in example 1, would actually give the false impression that disease severity (i.e., population threat) is worse than it actually is. Conversely, as in the second example, if one were not careful about their sampling design, they may not detect the disease at all or may report prevalences lower than they are in actuality (especially if they do not sample individuals from infection pockets). Both of the above scenarios illustrate the importance of conducting *Bd*

surveys along large transects in order to capture heterogeneity of disease prevalence within populations. They also illustrate the value of long term studies to accurately monitor host abundance and infection dynamics (especially if the pathogen is truly novel). Such strategies will provide more valuable information about *Bd* population effects than disease "snapshot" prevalence studies can alone. Additionally, they can provide more accurate estimates of population disease prevalence in dynamic and/or heterogeneous host populations.

I mention this here because I would like to emphasize that although I may not have sampled 30 individuals from every population, my sampling scheme was designed in order to minimize some of the potential problems mentioned above (e.g., many populations were sampled from the same environmental conditions, sampling procedure consisted of long transects). Finally, even though I believe that this study made a valuable contribution to science, by being the first to systematically investigate *Bd* prevalence across Peru, such an investigation is only providing a glimpse of what is actually going on with *Bd* in Peru; and the only way to actually understand the true effects of this disease in Peru is to implement long term studies.

Techniques for Detecting *Bd*

Since the discovery of *Bd* in 1998, several methods have been used for detection; among these PCR from skin swabs is accepted as the best method due to its high sensitivity, non-invasiveness and ease of use. However, since PCR is a chemical reaction that requires a specific DNA template to proceed, this method relies upon the presence of non-degraded DNA template. The methods discussed in Chapter 3 were developed with the hopes that they would increase the number of *Bd* studies being performed in developing countries or by researchers from smaller labs, which may not be able to afford qPCR equipment and reagents. I showed that the end-point PCR assay using Amplitaq Gold (ABI) that I developed is equally sensitive to the conventional *Bd* qPCR assay. I argued that in addition to the being cost prohibitive, the *Bd* qPCR assay has the added disadvantage of reduced specificity in comparison to end-point PCR. Lastly, I explained that the reduction in the specificity of qPCR is an inherent characteristic of this test that is driven by the occurrence of background fluorescence, which makes definitive scoring of presence/absence of *Bd* a subjective process.

I also tested two widely used methods for DNA extraction (DNeasy, Qiagen and PrepMan Ultra, ABI) and three methods for sample preservation (dry, 95% ethanol, and DMSO). My results showed that despite the extra cost and time commitment of performing the DNeasy technique, it outperforms PrepMan Ultra in both extraction efficiency and reliability. Additionally, I discussed the pros and cons of the three preservation methods that I tested and suggest that although all three the methods worked similarly well, the 95% ethanol method should be used for field studies since it has a lower risk of failure (i.e., because it eliminates the possibility of growth from microbial contaminants).

This study is the first to compare the many of the published methods for *Bd* sample preservation, extraction, and detection. I believe that the results of this study will benefit *Bd* research by providing a reliable and cost effective means of performing *Bd* research in smaller, less financially stable labs.

Minimizing PCR Inhibition

The occurrence PCR inhibition in *Bd* studies is frequently overlooked despite reports that it can occur in up to 25% and 72.5 % of samples (Hyatt et al. 2007, Garland et al. 2010 respectively). Reports of such high incidences of PCR inhibition suggest that researchers who do not nullify the effects of PCR inhibitors may be drastically underestimating *Bd* prevalence.

In Chapter 4, I showed that PCR inhibition affected at least 89% of samples tested, and proposed that the main cause of PCR inhibition in the *Bd* assay may be humic acid, a known inhibitor of Taq polymerase. I tested the effectiveness of eight techniques developed by researchers working with soil and fecal samples for dealing with humic acid inhibition. Four of techniques tested can be used for DNA extraction: DNeasy (Qiagen), QIAamp DNA Stool (Qiagen), PowerLyzer PowerSoil (MoBio), and PrepMan Ultra (ABI); and four techniques were developed for analyzing *Bd* samples post-DNA extraction: Amplitaq Gold (ABI), Bovine Serum Albumin (BSA, Sigma Aldrich), PowerClean DNA Clean-up Kit (MoBio), and inhibitor resistant Taq Polymerase mutants (DNA Polymerase Technologies).

All of the techniques tested were fairly good at removing humic acids, with the exception of PrepMan Ultra, which was only capable of removing 0.01 µg/µl of humic acid from the samples (interestingly this is the most widely used extraction method in *Bd* studies, of 42 studies analyzed, 88% used PrepMan Ultra, author unpublished). Because the techniques varied in effectiveness, I discussed the pros and cons of each method and conclude by recommending the use of the DNeasy kit for DNA extraction, and the end-point PCR assay with Amplitaq Gold and BSA due their effectiveness at removing humic acids, low cost, and relative ease of use. Additionally, although I found that PowerClean was very effective at removing humic acids, I recommend that it only be used for samples that are still inhibitory even after the

addition of BSA, as PowerClean is a very costly and time consuming method to perform. Lastly, I verified the use of these methods using real skin swabs collected in Peru in 2011. The addition of BSA to the end-point PCR with Amplitaq Gold improved detection capability by 4%, and showed that detection capability can be further increased by 8% when purifying samples with PowerClean and repeating the PCR. The dramatic increases in *Bd* detection capability observed when using BSA and/or PowerClean highlight the major consequences that humic acid contamination can have on *Bd* PCR assay results. These results indicate that studies that do not address humic acid contamination when performing DNA extraction and PCR analyses may be dramatically underestimating *Bd* prevalence.

Highlights and Broader Impacts

In this document I have emphasized the importance of investigating chytridiomycosis, and stressed the importance of conducting studies of this disease in undeveloped countries. I have also discussed the value of continued surveillance of amphibian populations and discussed some management strategies for *Bd*. The results of my investigation of the prevalence of *Bd* in Peru demonstrate that the disease is widespread throughout Peru. My discovery of a *Bd*-positive *Telmatobius marmoratus* at a market in Cusco provides further support to the developing hypothesis that amphibian trade may be abetting the global spread of *Bd*. Lastly, I have developed and verified the efficacy of several methods for sample preservation, DNA extraction, PCR testing, and reduction of PCR inhibition, which greatly improve applicability of performing *Bd* assays in smaller labs or in developing countries.

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APPENDIX A

Table A. Major supplies companies used for techniques discussed in this document.

Company	City	State
Applied Biosystems (ABI)	Foster City	CA
BioSpec Products	Bartlesville	OK
DNA Polymerase Technology, Inc.	St. Louis	MO
Fisher Scientific	Pittsburg	PA
Gene Tools	Philomath	OR
Genesee	San Diego	CA
Invitrogen	Grand Island	NY
Medical Wire and Equipment	Durham	NC
MoBio	Carlsbad	CA
Pisces Molecular	Boulder	CO
Promega	Madison	WI
Qiagen	Valencia	CA
Sigma-Aldrich	St. Louis	MO
USB Products	Cleveland	OH

Table B. Major supplies used for techniques compared in Chapter 3.

Product	Company	Product #	Amount	Cost
Chytr51 MGB TaqMan Probe	Applied Biosystems	4316034	6,000 pmoles	$255
PrepMan Ultra	Applied Biosystems	4318930	20 ml	$108
GeneAmp® dNTP Blend, 10mM	Applied Biosystems	N8080260	1 ml	$50
AmpliTaq Gold w/ Buffer and $MgCl_2$	Applied Biosystems	4311816	1000 units	$670
GeneAmp® dNTP Blend, 10mM	Applied Biosystems	N8080260	1 ml	$50
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems	N8010560	10 plates	$114
TaqMan® Exogenous Internal Positive Control Reagent	Applied Biosystems	4308323	200 rnx	$230
Zirconium/silica beads (0.5mm diameter)	BioSpec Products	11079105z	454 g	$47
Mini Beadbeater 1	BioSpec Products	3110BX	1 unit	$842
Optical Adhesive Covers	Applied Biosystems	4360954	25	$62
7500 Fast Real-Time PCR System	Applied Biosystems	4359286	1	$49,000
Polypropylene Microvials (for Beadbeater)	BioSpec Products	522S	500 count	$134
Sterile Dry Swabs	Medical Wire & Eq.	MW113	1250 swabs	$298
100 bp DNA ladder	Promega	PR-G2101	250 µl	$112
DNeasy Kit	Qiagen	69506	250 samples	$583
AlumSeal II Films	Genesee	12-169	100	$85
96-well PRC Plates	Genesee	24-300	25	$107
Peltier PTC-200 Thermal Cycler	Gene Tools	PTC-200	1	$3,800
ExoSAP-IT	USB	78200	200 µl	$96

Table C. Major supplies used for techniques compared in Chapter 4.

Product	Company	Product #	Amount	Cost
PrepMan Ultra	Applied Biosystems	4318930	20 ml	$108
GeneAmp® dNTP Blend, 10mM	Applied Biosystems	N8080260	1 ml	$50
AmpliTaq Gold w/ Buffer and $MgCl_2$	Applied Biosystems	4311816	1000 units	$670
Zirconium/silica beads (0.5mm diameter)	BioSpec Products	11079105z	454 g	$47
Mini Beadbeater 1	BioSpec Products	3110BX	1 unit	$842
Sterile Dry Swabs	Medical Wire & Eq.	MW113	1250 swabs	$298
100 bp DNA ladder	Promega	PR-G2101	250 µl	$112
DNeasy Kit	Qiagen	69506	250 samples	$583
QIAamp DNA Stool	Qiagen	51504	50 preps	$192
PowerLyzer PowerSoil	MoBio	12855-50	50 preps	$218
PowerClean DNA	MoBio	12877-50	50 preps	$166
Humic Acid	Sigma-Aldrich	53680	10 g	$40
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A4161	250 mg	$33
Omni Klentaq LA	DNA Poly. Tech	350	125 µl	$270
PCR Enhancer Cocktail-2	DNA Poly. Tech	E620	6,250 µl	$120

APPENDIX B

IACUC Approval Letters

EAST CAROLINA UNIVERSITY

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

December 18, 2008

Kyle Summers, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. Summers:

Your Animal Use Protocol entitled, "Chytridiomycosis in Peru," (AUP #D229) was reviewed by this institution's Animal Care and Use Committee on December 16, 2008. The following action was taken by the Committee:

"Approved as submitted"

As a note, the pending NSF grant has not been submitted to the IACUC for review.

Please contact me if I can be of further assistance.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

East Carolina University

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CENTENNIAL

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

November 18, 2009

The National Science Foundation
4201 Wilson Boulevard
Arlington, VA 22230

Dear Sir or Madam:

The following application submitted to the National Science Foundation was reviewed and approved by this institution's Animal Care and Use Committee:

Title of Application: "Investigation of the Distribution and Strain Variation of Chytridiomycosis in Peru"

Name of Principal Investigator: Kyle Summers, Ph.D.

Name of Institution: East Carolina University

Date of Approval: November 18, 2009

This institution is fully accredited by AAALAC and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare. The Assurance Number is A3469-01.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd