QUANTITATIVE, COMPETITIVE PCR AND FLUORESCENT MICROSCOPY METHODS FOR THE STUDY OF *BATRACHOCHYTRIUM DENDROBATIDIS*

BY

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ABBREVIATIONS

Bd: *Batrachochytrium dendrobatidis*

UV-B: ultraviolet B

DNA: deoxyribonucleic acid

PCR: polymerase chain reaction

QC-PCR: quantitative, competitive polymerase chain reaction

RT-PCR: real-time polymerase chain reaction

BSA: bovine serum albumin

LSM: laser scanning microscope

IgM: immunoglobulin M

IgX: immunoglobulin X

IgY: immunoglobulin Y

DIC: differential interference contrast

ABSTRACT

Chytridiomycosis is a disease affecting amphibian populations worldwide and is caused by the parasitic fungus *Batrachochytrium dendrobatidis* (*Bd*). The catastrophic decline in numbers of amphibians dictates the need for full characterization of the fungus and amphibian host responses. Significant areas of research that would benefit from strengthening available methods are diagnostic techniques and fundamental molecular biology. A quantitative, competitive PCR (QC-PCR) technique is described that will improve cost efficiency of collecting data on the quantification of *Bd* in infected animals and the environment. QC-PCR adapts conventional PCR reagents and a competitor DNA sequence into a quantitative technique. Fluorescent microscopy is a vital tool used to study cellular and molecular biology. DNA and cell wall fluorescent staining of viable *Bd* observed with laser scanning confocal microscopy is described and should provide the foundation for future studies of *Bd* involving fluorescent microscopy. Both QC-PCR and fluorescent microscopy are technique advancements that will contribute to future studies and to understanding of *Bd* and chytridiomycosis.

CHAPTER I

Worldwide amphibian declines began to alarm biologists in the 1980s and prompted greater study of amphibian populations and investigation into potential causative agents. The First World Congress of Herpetology in 1989 instigated greater awareness of adverse trends regarding amphibian populations. There are over 6400 amphibian species, and assessment of their numbers indicate a high occurrence of declines or extinctions. Hypotheses for this phenomenon include climate change, habitat destruction, introduction of non-native species, environmental chemicals, increased ultraviolet (UV-B) light, and infectious diseases (Alford and Richards, 1999; Rollins-Smith, 2009). Amphibian populations in Australia, Puerto Rico, Canada, Finland, and Britain declined following climate changes such as drought, hurricanes, or local alterations. These changes caused decreased prey availability, habitat alterations, or abnormal reproductive behavior leading to a reduction in amphibian numbers (Alford and Richards, 1999). Habitat destruction is a well-documented cause of declines resulting from logging or mining activities, wetland drainage, or changes in land use. These activities can eliminate feeding, breeding, and shelter locations for amphibians and fragment ecosystems. Introduction of non-native species can increase predation of another species as was seen in localized extinctions of frog populations following predatory fish introduction in Sierra Nevada lakes (Alford and Richards, 1999). North American bullfrogs (*Rana catesbeiana*) feed on smaller frogs and have been implicated in declines of native frog populations (Alford and Richards, 1999). Chemicals, particularly those causing acidification of amphibian environments, are believed to decrease embryonic and larval viability. Increased UV-B resulting from ozone depletion in the stratosphere also reduces embryonic viability by causing DNA damage. A DNA-repair enzyme, photolyase, was found to be less abundant in embryos of declining species from Oregon. Infectious agents that are known to cause amphibian mortality include *Aeromonas hydrophila*, *Ranaviruses*, and *Batrachochytrium dendrobatidis* (Alford and Richards, 1999; Longcore et. al., 1999).

In 1998, Berger et. al. identified a chytridiomycete fungus (Chytridiomycota, Chytridiales) correlating with mass mortalities in Australian and Central American anurans. Longcore et. al. isolated the fungus from a blue poison dart frog (*Dendrobates azureus*) and further characterized the fungus, giving it the name *Batrachochytrium dendrobatidis* (1999). The disease caused by *B. dendrobatidis* (*Bd*) became known as chytridiomycosis. Analysis of additional amphibian mortalities in correlation with environmental factors led to the hypothesis of climate-induced *Bd* emergence as the primary cause of amphibian declines, although this view has remained controversial (Daszak et. al., 2003; Pounds et. al., 2006). *Bd* is unusual within the order Chytridiomycetes in that it is the only member known to infect vertebrates (Berger et. al., 2005). Other chytridiomycetes are parasitic organisms of plants, invertebrate animals, algae, Protista, and other fungi. Chytridiomycetes are ubiquitous organisms found from arctic to tropical regions. Common habitats include ponds, lakes, and other aquatic environments, but they are also found in moist soils. The ability of these fungi to metabolize chitin, keratin, and cellulose makes this order important in the biodegradation of plant and animal remains (Powell, 1993).

Infection of amphibians by *Bd* occurs when the fungus penetrates amphibian skin and becomes an intracellular parasite within epidermal cells. Infection only occurs in regions of skin that are keratinized, prompting the hypothesis that keratinized skin is necessary for fungal infection. Amphibians develop keratinized skin as they mature; thus, adults are more susceptible to chytridiomycosis than tadpoles. Mouthparts of tadpoles are their only keratinized structures and can be infected with *Bd*. Clinical signs of severe chytridiomycosis include lethargy, inappetence, irregular skin sloughing, abnormal posture, and loss of righting reflex (Voyles et. al., 2011). Amphibian skin is important in osmoregulation and active electrolyte transport. Through an unknown mechanism, chytridiomycosis can slow electrolyte transport across the skin, which consequentially lowers potassium and sodium concentrations in the plasma. Depleted electrolyte concentrations result in asystolic cardiac arrest and mortality (Voyles et. al., 2009).

Zoospores are the earliest stage in the fungal life cycle and are motile by means of a single flagellum. Their typical size is 3-5 µm in diameter excluding the flagellum. Upon attachment to a surface, zoospores will encyst, reabsorb their flagellum, and grow branching rhizoids in a stage referred to as a germling. Further maturation leads to the zoosporangia stage, where mitotic division leads to multinucleate formation as the organism grows in size up to 40 µm. One or two discharge tubes or papillae will form and release mature zoospores in completion of the 4 to 5 day life cycle. When grown on amphibian skin, *Bd* zoospores will become intracellular within epidermal cells of the stratum corneum and complete their life cycle as described previously. Discharge tubes will protrude through the surface of the host cell and allow release of zoospores into the extracellular space (Berger et. al., 2005). Permissive growth conditions for *Bd* range in temperature from 4°-25°C and in pH from 4-8 (Piotrowski et. al., 2004). The life cycle of *Bd* is believed to be the same whether grown *in vitro* or *in vivo* (Berger et. al., 2005).

While the discovery of *Bd* as an infectious agent was only made just over a decade ago, chytridiomycosis is an unmatched amphibian disease in its potential impact on global amphibian populations. Retrospective studies indicate the presence of *Bd* in preserved amphibian specimens from South Africa from as early as 1938 (Weldon et. al., 2004). The origin of this fungus is believed to be in Africa; initial distribution to other regions likely occurred with the international trade of *Xenopus laevis*. This amphibian species was found to be useful in pregnancy tests and was shipped to numerous locations worldwide starting in 1935 (Weldon et. al., 2004). *X. laevis* is highly resistant and does not undergo mortality from chytridiomycosis, allowing it to be a carrier for *Bd*. Dispersal of *Bd* may also occur through trade of the American bullfrog, *Rana catesbeiana*; by a vector such as a bird, insect, or human; or through water (Kilpatrick et. al., 2009).

Due to the spread and impact of *Bd*, efforts to contain chytridiomycosis and sustain amphibian species are paramount for avoiding massive extinctions. In order to improve methods for the study of *Bd*, two methods are described in the following chapters that can be used in future

studies of mycological, infectious, and molecular aspects of *Bd* as well as quantitative detection of this fungus.

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CHAPTER II

Quantification of *Batrachochytrium dendrobatidis* by Quantitative, Competitive PCR

ABSTRACT

Effects of *Batrachochytrium dendrobatidis* (*Bd*) on amphibian populations worldwide have demonstrated the need for techniques not only for detection but also for quantification of this fungus. A quantitative, competitive polymerase chain reaction (QC-PCR) was developed and validated for use in quantifying *Bd*. For amplification of the endogenous *Bd* sequence, a previously published primer set that specifically amplifies a 260 bp DNA sequence within the ITS-1 and ITS-2 region of *Bd* was used. The competitor sequence was an internal sequence from *Drosophila melanogaster* and was modified to contain identical primer-binding sites. The competitor was shown to have similar amplification efficiency as the *Bd* target sequence and was competitive when co-amplified with the target sequence. Sensitivity of this method was demonstrated in water samples with known numbers of *Bd* zoospores processed through membrane filtration. A standard curve was developed for the quantification of 10 to 100,000 zoospores in water samples.

INTRODUCTION

Chytridiomycosis is an infectious disease caused by *Batrachochytrium dendrobatidis* (*Bd*) and is responsible for widespread amphibian mortalities (Kilpatrick et. al., 2009). *Bd* was first identified in the late 1990s (Berger et. al., 1998 and Longcore et. al., 1999) but has been observed in preserved amphibian specimens from South Africa from as early as 1938 (Weldon et. al., 2004). Recent evidence suggests that amphibian mortality correlates with disrupted transport of electrolytes across the skin, thereby inducing asystolic cardiac arrest as a result of decreased plasma sodium and potassium concentrations (Voyles et. al., 2009). While ecological studies have documented the prevalence of this fungus and the correlation to mass mortalities in amphibians, understanding of the transmission dynamics, infection mechanisms, and host immune response are limited.

Techniques available for detecting *Bd* have become more sensitive as researchers have utilized various molecular methods. Early methods of detecting *Bd* were limited to histological examinations, electron microscopy, and culture conditions (Berger et. al., 1998; Berger et. al., 2002). The validation of polymerase chain reaction (PCR) methods has provided the current preferred assay for the qualitative assessment of *Bd* infection (Annis et. al., 2004; Boyle et. al., 2004). Adaptation of PCR into a semi-quantitative method for enumeration of *Bd* by utilization of real-time PCR (qRT-PCR) provides a way to gauge the number of fungi in samples from an infected amphibian or an environmental (soil, water, etc.) sample (Kriger et. al., 2006a; Kirshtein et. al., 2007). qRT-PCR has been thoroughly validated for use in field studies and laboratory infections and has become the primary assay for quantitative detection of *Bd* (Kriger et al., 2006a; Hyatt et. al., 2007). Whereas qRT-PCR offers the benefits of specificity and semi-quantitative analysis, its use requires a real-time thermal cycler and fluorescent probes that may make it prohibitively expensive for a number of uses. Reduction of reagent volumes used and other modifications have been made to improve cost efficiency of qRT-PCR (Kriger et. al., 2006b).

Even with these attempts to reduce the cost of qRT-PCR, conventional PCR is still less expensive and equally sensitive at detecting *Bd* (Garland et. al., 2011).

Although qRT-PCR is regarded as the preferred method for quantification of *Bd*, the practical considerations of cost and the need of a real-time thermal cycler can limit its widespread use. Quantitative, competitive PCR (QC-PCR) is a technique that provides fully quantitative data, but its use has not been previously described with *Bd*. Standard conventional PCR reagents and conditions are used in QC-PCR, but a competitor sequence is added into the amplification reaction and acts as an internal standard. Co-amplification of samples containing the target DNA sequence, with known concentrations of the competitor sequence added, will generate amplicons equal in ratio to the number of both starting DNA sequences. Thus, when the number of amplicons produced is equal, the target DNA sequence can be equated to the known concentration of the competitor DNA sequence. This method has been used successfully in the quantification of bacteria, fungi, and eukaryotic parasites in a number of experimental systems (Qiu et. al., 2004; Brunk et. al., 2002; Gobbin et. al., 2007; Piña-Vázquez et. al., 2008; Sekhavati et. al., 2009). Modification of a conventional PCR technique into a QC-PCR technique requires the construction of a competitor sequence with identical primer-binding sites as the target sequence and comparable amplification efficiencies of the competitor and target sequence (Zimmermann and Mannhalter, 1996). Starting concentrations of target DNA sequences can be calculated by comparison with the internal standard. Alternatively, a standard curve generated from samples with known numbers of organisms can be used to calculate organisms within a test sample. Variations due to sample processing efficiencies are minimized by maintaining identical processing procedures between generation of the standard curve and unknown samples.

Using QC-PCR may provide field and laboratory researchers with a cost effective means of quantifying *Bd*, either by calculating the number of starting target *Bd* DNA sequences within a sample based on the amplified target DNA/competitor DNA ratios or by calculating the number

of organisms based on a standard curve. A QC-PCR method is described herein that may be useful in both water samples and tissue samples.

MATERIALS AND METHODS

Competitor Sequence Construction. The competitor sequence was designed to contain *Bd*1a and *Bd*2a primer binding sites identical to those found in the ITS-1 and ITS-2 regions of *Bd* (Annis et. al., 2004). Based upon size similarity and availability, the sequence *CG6986* encoding the proctolin receptor in *Drosophila melanogaster* was selected to provide the internal competitor sequence (Johnson et al., 2003). Hybrid primers consisting of the forward and reverse *CG6986* primers, with a 5' addition of 12 bp from the 3' ends of the *Bd*1a and *Bd*2a primers, were designed (Table 1) and used to amplify and extend the internal sequence. Following 1.0% agarose gel electrophoresis and DNA purification with Qiaex II gel extraction kit (Qiagen), the resulting DNA sequence was further extended and amplified with *Bd*1a and *Bd*2a primers to yield a 237 bp product.

PCR Conditions. PCR conditions were based on those used by Annis et al. (2004), but were shortened to reduce running time and adjusted for the optimal temperature of the primers. A hot start of 5 min. at 93°C was followed by 40 cycles (unless otherwise noted) of 93°C for 30 sec., 48°C for 20 sec., and 72°C for 20 sec., with a final extension of 5 min. at 72°C. *Bd*1a and *Bd*2a primers were used at concentrations of 700 µM apiece. Also included were 1X ExTaq DNA polymerase buffer (Promega, Madison, Wisconsin), 0.2 mM of each dNTP (Promega, Madison, Wisconsin), 400nM bovine serum albumin (BSA; Sigma, St. Louis, Missouri), and 0.625 U of ExTaq DNA polymerase (Promega, Madison, Wisconsin) in a total reaction volume of 50 µl. The use of BSA in PCR had been independently observed to reduce inhibition and has since been validated (Garland et. al., 2009). PCR was performed in either an Eppendorf Mastercycler or Bio-Rad Peltier Thermal Cycler 200.

Validation of Competitive Amplification. Samples of both *Bd* target and competitor DNA were purified using the Qiaex II gel extraction kit (Qiagen, Valencia, California). To ensure that both sequences of DNA had similar rates of PCR amplification efficiency, equal molar concentrations corresponding to 10^8 individual sequences of purified *Bd* and competitor

DNA were mixed and co-amplified using the previously described PCR conditions for a total of 30 cycles. PCR products were collected every second cycle following the 14th cycle; they were run on a 1.8% agarose gel and visualized with ethidium bromide and a Hitachi GeneSnap imager and software. Raw fluorescent intensities of *Bd* and competitor amplicon bands in the gel were measured. To derive a ratio of *Bd* to competitor sequences from raw band intensities, correcting for unequal sequence sizes was necessary. Multiplying *Bd* band intensity by 237bp/260bp ensured that fluorescent intensity would not be falsely elevated due to the larger size of the *Bd* target sequence.

Organisms and Culture Conditions. *Bd* strain 423 was obtained from Dr. Joyce Longcore (University of Maine) and was cultured on 1% agar containing 1% tryptone media at room temperature. Zoospores were harvested by rinsing plates with either sterile distilled water or 1% tryptone. Dilutions of harvested zoospores in distilled water were made and counted on a hemocytometer when quantified numbers of zoospores were required.

***Bd* Collection and DNA Extraction by Membrane Filtration.** Samples of zoospores were prepared by adding a known quantity of newly-released zoospores into a total volume of 250 ml of distilled water. The entire volume was vacuum filtered using sterile 0.4-µm polycarbonate filters (Millipore, Billerica, Massachusetts) and a Nalgene filtration system using 47-mm membrane filters. Flow rate through the 0.4-µm filters ranged from 25 to 50 ml/min. The 0.4-µm filters were transferred face up into sterile 60-mm Petri dishes for DNA extraction. DNA was extracted from the polycarbonate filter in a method modified from Peccia and Hernandez (2006). Briefly, 1.5 ml of elution solution (200 mM Tris, 20 mM EDTA, 0.3% SDS, pH 8.0) was added to each Petri dish containing a face up 0.4-µm polycarbonate filter, and the Petri dishes and filters were shaken on a mechanical rotator at 100 rpm for 20 hours at 22°C. A 750-µl aliquot was then transferred to a sterile 1.5-mL Eppendorf tube, and two cycles of freezing and thawing (-80°C to 100°C) were performed. Finally, DNA was extracted by a standard

phenol:chloroform method and reconstituted in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA).

RESULTS

Competitive amplification. It was necessary to establish that the efficiencies of PCR amplification of both the target *Bd* sequence and the competitor sequence were comparable. Co-amplification of equal molar concentrations of purified competitor and target *Bd* DNA demonstrated equivalent amplification rates as well as similar patterns of exponential, linear, and plateau phases (Fig. 1). When serial dilutions of either target *Bd* or competitor DNA were co-amplified with a constant concentration of the other sequence, the ratios of amplified products yielded a linear relationship given proximal starting concentrations of DNA. This linear relationship provides the basis for quantifying target *Bd* sequences based on known concentrations of the competitor sequence.

Quantification of *Bd* following membrane filtration. Filtration and DNA extraction of distilled water samples containing known concentrations of *Bd* zoospores yielded positive conventional PCR amplification for as few as 10 zoospores in a 250-ml starting sample. Samples containing from 10 to 100,000 zoospores were processed through filtration, DNA extraction, and qualitative PCR. Samples containing *Bd* were then co-amplified with multiple concentrations of competitor sequence from a 1:2.5 serial dilution of purified competitor DNA. Fluorescent intensities of target *Bd* products and competitor PCR products were recorded from agarose gels stained with ethidium bromide and transformed into log ratios (Fig. 2A). For each sample, co-amplification reactions that produced visible target and competitor products were used to generate a graph of logarithmic intensity ratios (Fig. 2A). The point at which the fitted linear regression line has a Y-value equal to 0 is the point at which the concentrations of competitor and target *Bd* amplicons are calculated to be equal. Graphing the relationship of the X-values where Y=0 for all water samples containing from 10 to 100,000 *Bd* zoospores demonstrated a power regression line with an R^2 value of 0.9982 (Fig. 2B).

DISCUSSION

Design and validation of this QC-PCR method for sensitive, specific quantification of *Bd* required (1) a competitor DNA sequence with PCR amplification efficiency comparable to the target *Bd* sequence, (2) primer sets specific for detecting *Bd,* and (3) data that demonstrated QC-PCR sensitivity in a practical application. The use of *Bd*1a and *Bd*2a primers (Annis et. al., 2004) that had previously been tested for specificity eliminated the necessity of testing for primer specificity.

The constructed competitor sequence was demonstrated to amplify at the same efficiency as the target *Bd* sequence. When samples of purified target *Bd* and competitor DNA sequences were co-amplified, keeping the concentration of one constant and serially diluting the other, the ratios of resulting products demonstrated a linear relationship for samples with approximately equal starting DNA concentrations. Co-amplification of both sequences and the resulting amplification curves yielded matching PCR phases. The observed linear relationship from co-amplifications with serial dilutions demonstrates comparable amplification efficiency between the target *Bd* and competitor sequences.

Sensitivity in a practical application was tested on samples from membrane filtration of water containing *Bd*. The membrane filtration method was shown to qualitatively detect as few as 10 zoospores in 250 ml of distilled water. QC-PCR testing of samples containing 10 to 100,000 zoospores produced results that provided a standard curve for testing unknown samples processed in the same manner. The DNA extraction method described herein eliminates the need for commercial DNA extraction solutions, which are often needed in large quantities when used with membrane filters.

In field studies, whether or not a body of water contains *Bd* or if the amphibian species within it are infected by *Bd* are vital statistics. Quantitative data allow monitoring seasonal variation of *Bd* concentrations or infection levels more precisely than qualitative data. Researchers focusing on field studies may benefit from the precision that QC-PCR provides,

without the expenses associated with RT-PCR. Studies focusing on dynamics of *Bd* infection or host immune responses rely upon monitoring the infection load of the host. QC-PCR can be used in water, swab, or tissue samples in a reproducible manner.

In conclusion, QC-PCR has been validated as an alternative technique for the quantification of *Bd*. This tool may allow broader access to more quantitative data for *Bd* researchers. Given the global nature of this fungus, a choice in available molecular methods may provide a means to increase understanding of the nature and transmission of *Bd*.

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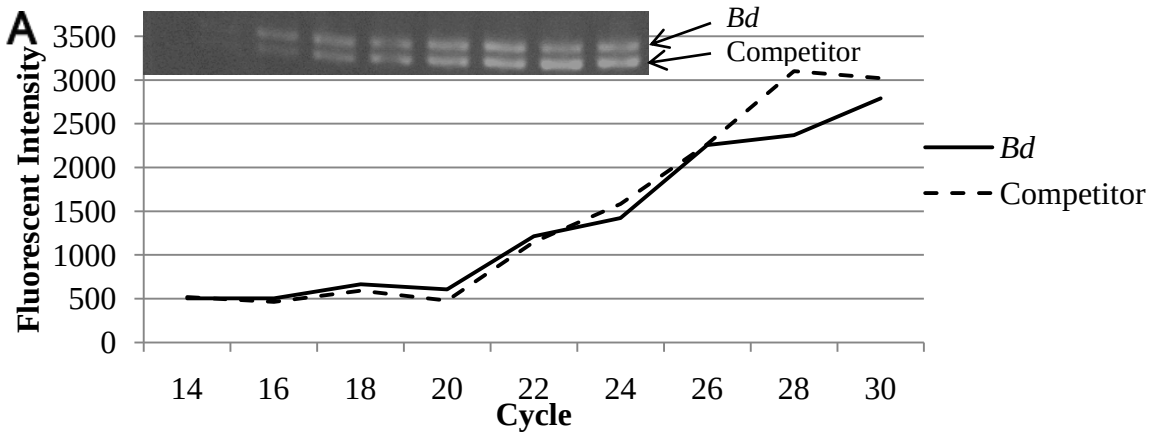
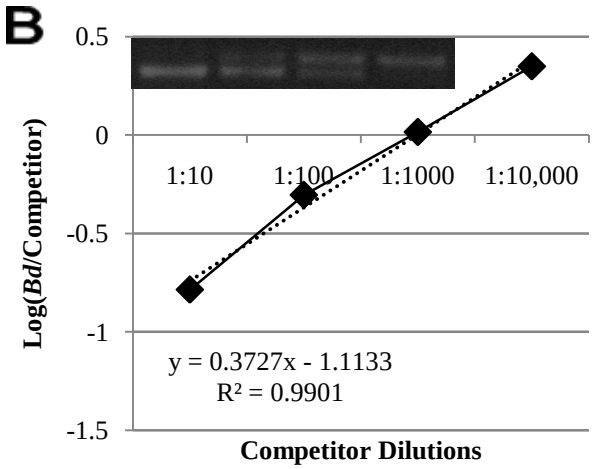
Table 1: Primers used for internal standard construction.

CG6986 FP	5’-TACGGATATTGCCTACCTGACGTGCCA-3’
CG6986 RP	5’-ACACACATTTCCCGCCACCACCTGAGTG-3’
Hybrid Primer 1	5’-*CCATATGTCACG* TACGGATATTGCCTACCTGACGTGCCA-3’
Hybrid Primer 2	5’-*TATCTGTCCAG* ACACACATTTCCCGCCACCACCTGAGTG-3’
*Bd*1a	5’-CAGTGTG *CCATATGTCACG*-3’
*Bd*2a	5’-CATGGTTC *ATATCTGTCCAG*-3’

Figure 1: *Batrachochytrium dendrobatidis*. Co-amplification of *Bd* target and competitor DNA. (A) Agarose gel band intensities of *Bd* and competitor co-amplification products from starting molar concentrations of 10^8 sequences of both. (B) Logarithmic ratios of band fluorescent intensities of target *Bd* sequences co-amplified with serial dilutions of competitor sequence. A regression line was fitted to the data and demonstrated that the ratio of amplicons was equal to the starting ratio of concentrations of both sequences. (C) Logarithmic ratios of band fluorescent intensities of competitor sequences co-amplified with serial dilutions of target *Bd* sequences. A regression line was fitted to this data and confirmed equal amplification efficiency between sequences.


A
Bd
Competitor
Fluorescent Intensity
3500
3000
2500
2000
1500
1000
500
0
14
16
18
20
22
24
26
28
30
Cycle
Bd
Competitor


B
0.5
0
-0.5
-1
-1.5
1:10
1:100
1:1000
1:10,000
Log(Bd/Competitor)
y = 0.3727x - 1.1133
R² = 0.9901
Competitor Dilutions

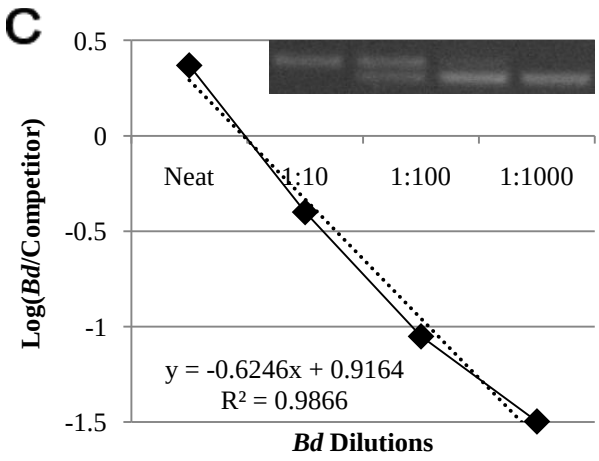
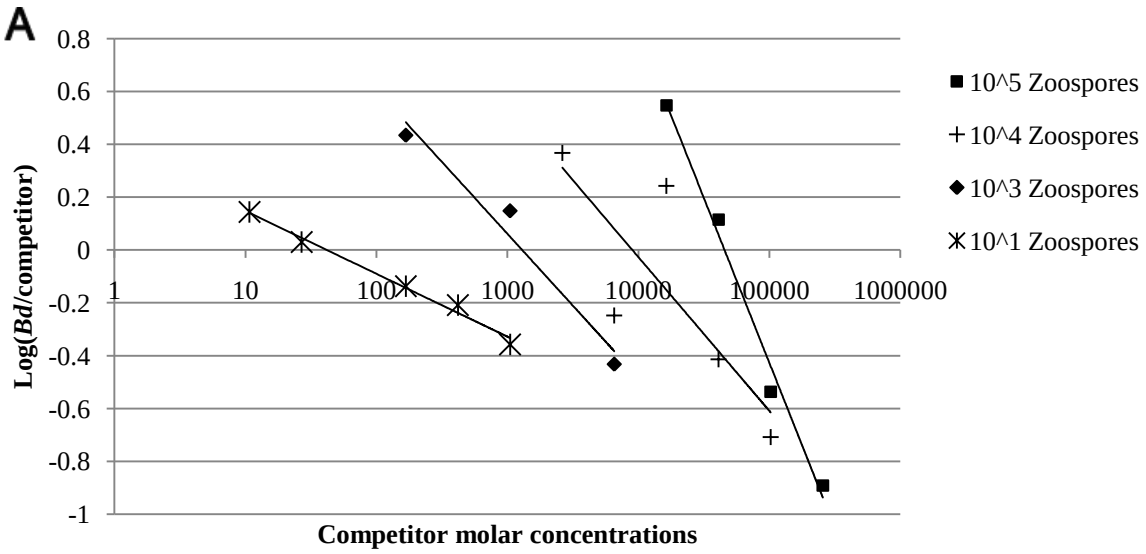
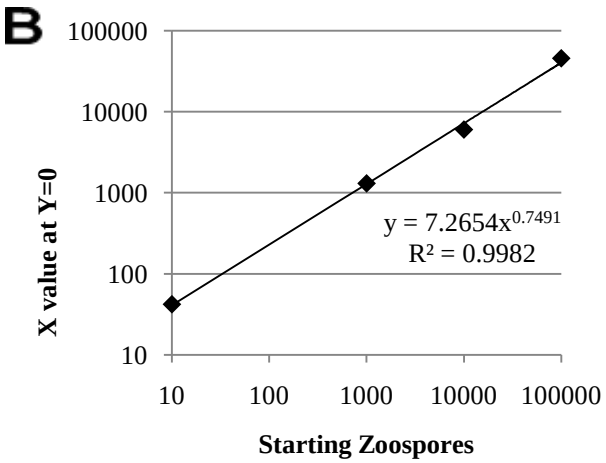

C
0.5
0
-0.5
-1
-1.5
Neat
1:10
1:100
1:1000
Log(Bd/Competitor)
y = -0.6246x + 0.9164
R² = 0.9866
Bd Dilutions

Figure 2: *Batrachochytrium dendrobatidis*. Quantification of known concentrations of zoospores and generation of standard curve. (A) Logarithmic ratios of target *Bd* and competitor co-amplification products. Distilled water samples containing known concentrations of zoospores were membrane filtered, and DNA was extracted. Individual samples were co-amplified with multiple concentrations of competitor DNA from a serial dilution. Fluorescent band intensities from an agarose gel were used to calculate *Bd* to competitor ratio. (B) Standard curve generated for distilled water samples of unknown *Bd* concentration. X-values where Y=0 from (A) were graphed with starting concentration of *Bd* zoospores.


A
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
1
10
100
1000
10000
100000
1000000
Log(Bd/competitor)
Competitor molar concentrations
10^5 Zoospores
10^4 Zoospores
10^3 Zoospores
10^1 Zoospores


B
100000
10000
1000
100
10
10
100
1000
10000
100000
X value at Y=0
Starting Zoospores
y = 7.2654x^0.7491
R² = 0.9982

CHAPTER III

Fluorescent Microscopy of Viable *Batrachochytrium dendrobatidis*

ABSTRACT

Batrachochytrium dendrobatidis (*Bd*), a chytrid fungus, is the causative agent of chytridiomycosis and amphibian population declines worldwide. The sequenced genome of *Bd* provides information necessary for studying the fungus and its molecular biology. Fluorescent microscopy is a technique used to image targeted molecules in live or fixed organisms to understand cellular trafficking and localization, but the use of fluorescent microscopy with *Bd* has not yet been demonstated. Two fluorescent stains were tested for their use in live cell imaging of *Bd*, the cell wall-specific fluorophore Solophenyl Flavine 7GFE and the DNA-specific fluorophore DRAQ5. These specific staining patterns were observed in live cultures of *Bd* when visualized with laser-scanning confocal microscopy. A *Rana pipiens* exposed to stained zoospores was euthanized four days after exposure and skin samples were found to contain fluorescent organisms resembling *Bd*. Based on these observations, fluorescent microscopy is a powerful tool that can be utilized in future studies of chytridiomycosis and the molecular and cellular biology of *Bd*.

INTRODUCTION

Chytridiomycosis is an infectious disease caused by *Batrachochytrium dendrobatidis* (*Bd*) and is responsible for widespread amphibian mortalities (Kilpatrick et. al., 2009). *Bd* is a parasitic fungus that was first identified in the late 1990s (Berger et. al., 1998 and Longcore et. al., 1999) but has been found in preserved amphibian specimens from South Africa from as early as 1938 (Weldon et. al., 2004). Recent evidence suggests that amphibian mortality correlates with the disrupted transport of electrolytes across the skin, thereby inducing asystolic cardiac arrest as the result of decreased plasma sodium and potassium concentrations (Voyles et. al., 2009). Due to its recent discovery, basic biological characteristics of *Bd* have not been studied to the extent needed to fully understand chytridiomycosis.

Microscopy techniques are essential to the study of fungal and parasitic organisms. A variety of microscopic techniques are available to researchers for imaging the structure and molecules of live cells (Stephens and Allan, 2003). *Bd* has been studied using traditional light microscopy, scanning electron microscopy (SEM), and transmitted electron microscopy (TEM). Both SEM and TEM are capable of high magnification images, but can only be used with fixed samples. Visualizing live *Bd* has been restricted to basic light microscopic methods with inherently limited resolution. Adapting a more advanced microscopic technique capable of live-cell imaging for the study of *Bd* would provide an additional resource for researchers.

Live cell fluorescent microscopy has revolutionized research of molecular and cellular biology with its capabilities of imaging localization of specific molecules within cells. Fluorophores, such as fluorescent proteins, fluorescent stains, and quantum dots, are molecules that emit light within a range of wavelengths following exposure to a specific excitation wavelength. The use of fluorescent microscopy in other fungal organisms has provided information on infection processes, vesicle formation and movement, and fungal morphology and growth (Kurtti and Keyhani, 2008; Saito et. al., 2004; Pitt et. al., 2004). With the goal of developing a foundation on which to design future fluorescent microscopy experiments,

fluorescent stains were selected and evaluated for their ability to target the fungal cell wall as well as intracellular DNA. Calcofluor white (Fluorescent Brightener 28; Sigma, St. Louis, Missouri) is a fluorescent stain that has been commonly used for its selective binding to chitin and cellulose in the cell walls of plants and fungi (Rasconi et. al., 2009). Excitation of calcofluor white, however, requires ultraviolet light, which causes phototoxicity and limits the use of laser-scanning confocal microscopy. Alternatives to calcofluor white have been identified, and two dyes (Solophenyl Flavine 7GFE 500 and Pontamine Fast Scarlet 4B) met the criteria of use with confocal microscopy without cellular toxicity (Hoch et. al., 2005). Imaging of DNA in viable cells is used to track localization and division and can be done with various commercially available DNA stains. The selected DNA stain used in the present study was DRAQ5, which specifically binds double-stranded DNA (Smith et. al., 2000; Martin et. al., 2005).

The purpose of this study was to demonstrate fluorescent microscopy of *Bd* as a tool for future studies in fields of mycology, molecular biology, host specificity, and others. In addition, the fluorescent stains Solophenyl Flavine and DRAQ5 were shown to have specific staining patterns in *Bd* for the fungal cell wall and DNA, respectively.

MATERIALS AND METHODS

Organisms and culture conditions

Bd strain 423 was obtained from Dr. Joyce Longcore (University of Maine) and was cultured on 1% agar containing 1% tryptone at room temperature (19°-22°C) routinely. Zoospores were harvested by rinsing three to five day old cultures with distilled water or 1% tryptone. Dilutions of harvested zoospores were counted using a hemocytometer. Cultures for confocal imaging were prepared by mixing freshly-released zoospores with fluorescent stains in 1% tryptone. Cultures were incubated for thirty min. in Eppendorf tubes at room temperature to allow uptake of fluorescent stains prior to transfer to 35-mm diameter plastic Petri dishes with #1.5 untreated glass cover slip bottoms (MatTek, Ashland, Massachusetts). Petri dishes were sealed with parafilm to prevent evaporation or contamination.

Validation of fluorescent specificity

Fluorescent specificity was tested by imaging fungal cells in cultures containing one stain only and observing emitted light within the range of wavelengths expected for both stains. Cultures containing two million *Bd* zoospores in either 1% tryptone with 0.0001% Solophenyl Flavine (Huntsman LLC, High Point, North Carolina) or 1% tryptone with 1 µM DRAQ5 (Biostatus Limited, Leicestershire, United Kingdom) were prepared to final volumes of 1.5 ml and incubated for thirty minutes at room temperature. Following incubation, the cultures were transferred to cover slip-bottomed Petri dishes. Cultures were imaged using identical excitation and emission settings (lasers, laser power, gain, etc.) and the presence and patterns of emitted fluorescence were compared.

Dual fluorescent staining of *Bd*

Use of Solophenyl Flavine and DRAQ5 together in stained cultures was tested to demonstrate imaging *Bd* with more than one fluorescent marker and to examine staining patterns of *Bd*. A 1.5-ml culture was prepared containing two million *Bd* zoospores in 1% tryptone containing 0.0001% Solophenyl Flavine and 1 µM DRAQ5. The cultures were added to cover

slip-bottomed Petri dishes following a thirty min. incubation and imaged once or twice daily for the following four days to observe emitted fluorescence of both stains and developmental changes of the organisms.

To test the possibilities of using stained *Bd* zoospores in infected amphibians, an adult *Rana pipiens* was exposed to fluorophore-labeled *Bd* zoospores. A zoospore suspension of two million *Bd* zoospores in 1% tryptone containing 0.0001% Solophenyl Flavine and 1 µM DRAQ5, with a total volume of 1.5 ml, was used as the inoculum. Prior to exposure, the zoospore suspension was labeled for thirty min. at room temperature in an Eppendorf tube and then centrifuged for 5 min. at 500g. The supernatant was removed to eliminate any fluorescent stain that had not bound to zoospores and was replaced with an equal volume of 1% tryptone. The zoospore pellet was resuspended by vortexing and then added to 250 ml of distilled water in the container housing a single adult *Rana pipiens*. On the fourth day following this exposure, the frog was killed and skin cross sections were made with a sterile razor blade and placed on glass slides with #1.5 cover slips and imaged immediately. Skin samples were examined for fluorescence of both Solophenyl Flavine and DRAQ5 in regions exemplifying *Bd* infection.

Confocal microscopy

A Zeiss LSM 710 single-photon confocal microscope was used with a 34-channel spectral detector (Carl Zeiss MicroImaging, Thornwood, New York). Excitation of Solophenyl Flavine was performed with a 405 nm laser line ranging in power from 0.004 to 1.0%. Excitation of DRAQ5 was achieved with a 633 nm laser line at 5.0 to 15.0% power. Emitted light was captured for Solophenyl Flavine from 410-600 nm and for DRAQ5 from 650-750 nm. A multi-track configuration was used to avoid possible excitation crosstalk and emission bleed through between fluorescent stains. The pinhole was set at or near 1.0 airy units for image acquisition. Transmitted light images were acquired simultaneously with a photomultiplier tube, although the plastic lid of the Petri dishes prohibited use of differential interference contrast (DIC). A 40x Plan-Apochromat dry objective with a numerical aperture of 0.95 was used for *in vitro* cultures

with a digital zoom of up to 7.5. For skin cross sections, a 20x Plan-Apochromat dry objective with a numerical aperture of 0.80 was used. Zen 2010 software was used for image acquisition. Image processing was performed by digitally filtering all images using the Adobe Photoshop (San Jose, California) unsharp mask tool.

RESULTS

In cultures stained with either DRAQ5 or Solophenyl Flavine, fluorescence was only observed in the emission ranges corresponding to the stain used (Fig. 1). DRAQ5 fluorescence was concentrated near the center of the cell for *Bd* germlings. In more mature zoosporangia, the fluorescence was shifted slightly off-center in conjunction with the location and size of the intracellular vacuole. Solophenyl Flavine fluorescence was present in the fungal cell wall, with greater intensity in zoosporangia than in germlings or zoospores. Rhizoids were also stained with Solophenyl Flavine but exhibited less fluorescence than the cell wall.

Dual-staining of cultures with DRAQ5 and Solophenyl Flavine demonstrated aforementioned staining patterns without any observed crosstalk between fluorescent stains. Cultures matured at the same rate as unstained cultures without noticeable morphological differences. Periodic imaging of a dual-stained culture provided fluorescent images of the maturation stages of *Bd* (Fig. 2). In the most mature stage, numerous distinct localizations of DRAQ5 staining indicate the presence of DNA in nuclei of new zoospores prior to release from a zoosporangium.

When an individual *Rana pipiens* was exposed to dual-stained *Bd*, cross sections of skin from the ventral side of the frog were observed to contain Solophenyl Flavine stained organisms at four days post-exposure (Fig. 3). DRAQ5 staining was not observed, possibly due to insufficient binding to zoospores and removal in the supernatant during the labeling step. Stained organisms did not appear beyond the superficial layer of skin. The frog had not developed clinical signs of chytridiomycosis at the time it was euthanized for sample collection.

DISCUSSION

The fluorescent stains Solophenyl Flavine and DRAQ5 are shown to be effective in binding to the *Bd* fungal cell wall and DNA respectively. Observed staining patterns demonstrate DRAQ5 localization in the nucleus and Solophenyl Flavine localization in the cell wall. These stains have been validated for use in other live-cell fluorescent imaging experiments and did not cause any observed toxic effects on *Bd*. Viability of *Bd* cultures in the presence of both stains did not appear to be diminished; zoospores retained motility and mature zoosporangia continued to form and release new zoospores at a corresponding manner and rate to unstained cultures. Stained zoospores appeared on the skin of a frog exposed to previously stained *Bd* zoospores, indicating that they retain the ability to bind to a host and presumably infect it. Solophenyl Flavine demonstrated strong fluorescence even with low laser power and did not demonstrate photobleaching. Because a 405-nm laser was used, phototoxic effects may be a problem for experiments requiring continuous imaging, although the 405-nm laser power can be set below 1% and still produce ideal fluorescent intensity. In a time-lapse experiment, growth and maturation of *Bd* germlings in the field of view appeared to slow or halt during the first six hours of image capture at half-hour time points, while *Bd* organisms outside the field of view matured normally. Visualization of DRAQ5 fluorescence was not observed in the skin samples taken from the exposed frog, either due to slow stain uptake by *Bd* and removal in the supernatant or insufficient stain concentration. Effects that DRAQ5 has in displacing DNA-binding proteins have been noted and related to corresponding cellular functions, suggesting caution in the interpretation of chromosome-related live cell data in future studies (Mari et. al., 2010). While transmitted light was captured in all images, the use of differential interference contrast (DIC) was prohibited by the plastic Petri dish lid for *in vitro* cultures. Imaging samples that avoid the use of plastic within the light path would prevent capturing unpolarized light and allow for higher contrast light microscopy images using DIC.

A secondary observation made during this study was the adhesive nature of *Bd* zoospores. Whether stained or unstained, *Bd* zoospores in liquid culture exhibited binding to untreated glass, some types of plastic, hair, and cotton fibers. These binding interactions could occur within ten minutes of initial contact. Binding to glass appeared to weaken as zoosporangia prepared to release new zoospores three to four days after culture. Future studies on the how this binding affects host specificity or infection mechanisms are needed, as adhesion is critical for fungal pathogens (Braun and Howard, 1994). A prominent cellular component of *Bd* observed during microscopy was a large vacuole that diminished in size as new zoospores developed within a zoosporangia. This vacuole remained free of DNA staining and appeared to displace the nucleus to near the cell wall. Vacuolar contents and functions in *Bd* have not been characterized yet, but storage of metabolites or proteases may be involved (Klionsky et. al., 1990; Veses et. al., 2008).

Imaging targeted fluorescent molecules in viable *Bd* will provide opportunities for the study of cellular biology in the forms of protein interactions, localizations, and DNA replication. The use of stained zoospores in experimental infections provides an option for observing fungal location within the skin of an infected amphibian without histological staining or specific antibodies. Adapting other fluorescent markers for use with *Bd* will continue to improve the methods available for the study of chytridiomycosis.

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Figure 1: *Batrachochytrium dendrobatidis* fluorescent specificity. Cultures containing two million zoospores in either 1% tryptone with 0.0001% Solophenyl Flavine or 1% tryptone with 1 µM DRAQ5 were placed in Petri dishes with coverslip bottoms and imaged using identical settings. Emitted light from 410-600 nm is pseudo-colored blue and emitted light from 650-750 nm is pseudo-colored red. A) Solophenyl Flavine emission in a DRAQ5 stained culture. B) DRAQ5 emission in a DRAQ5 stained culture. C) Solophenyl Flavine emission in a Solophenyl stained culture. D) DRAQ5 emission in a Solophenyl Flavine stained culture. Scale bars are 5 microns.

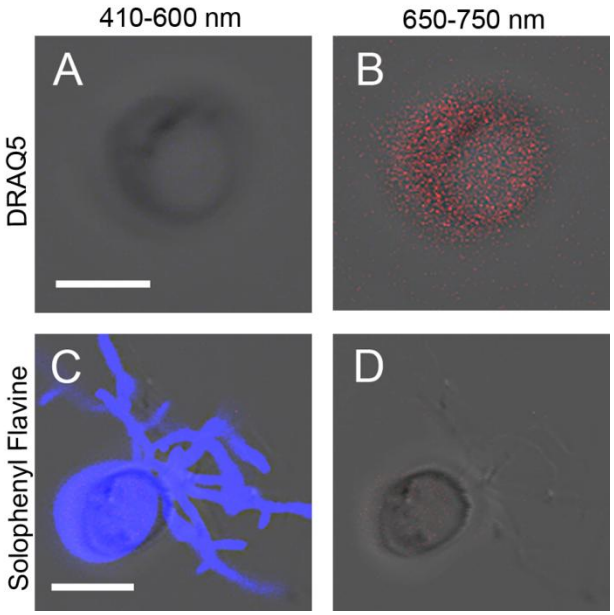

410-600 nm
650-750 nm
A
B
C
D
DRAQ5
Solophenyl Flavine

Figure 2: *Batrachochytrium dendrobatidis*. Merged confocal microscopy images of organisms in culture demonstrating Solophenyl Flavine and DRAQ5 staining patterns in stages from germling to zoosporangium. A culture of two million stained zoospores was added to a Petri dish and imaged periodically for the following four days to demonstrate staining patterns coinciding with culture growth. Scale bar is 5 microns; all images are at same magnification.

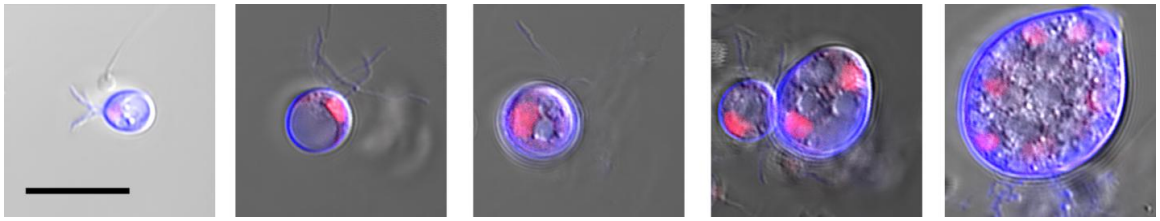
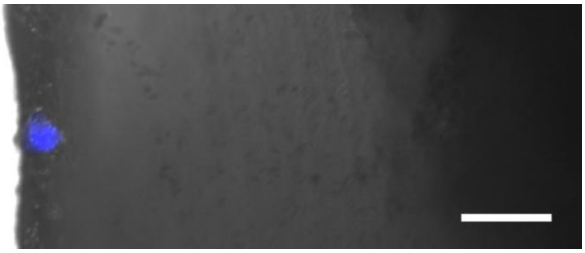


Figure 3: *Batrachochytrium dendrobatidis*. Confocal image of cross section of skin from a *Rana pipiens* exposed to two million *Bd* zoospores stained with 0.0001% Solophenyl Flavine and 1µM DRAQ5. Frog was exposed to *Bd* for 4 days prior to sample collection. Scale bar is 5 microns.



CHAPTER IV

The study of topics relating to *Bd* has progressed slowly due to the lack of research done on the Chytridiomycetes class of fungi in the years prior to *Bd* identification. Application of modern technologies and methods to the study of *Bd* has been hindered by the lack of precedence in related species and, until recently, lack of scientists studying *Bd* or chytridiomycosis. The presence of numerous characteristics found in other Chytidiomycetes remain poorly described in *Bd*. For example, host specificity is exhibited by some of the parasitic Chytridiomycetes (Powell, 1993). Although this feature could dictate effects on amphibian populations, *Bd* host specificity has not been tested. Chemotaxis of *Bd* toward nutrients or other chemoattractants, which would appear straightforward to test experimentally, has only been studied minimally with controversial results (Moss et. al., 2008; Piotrowski et. al., 2004). Other Chytridiomycetes have been identified as vectors for viruses that are pathogenic to the host of the fungus (Powell, 1993). Similar research relating to *Bd* has yet to be accomplished. These and other deficiencies are examples of gaps in the knowledge of *Bd*. Application of appropriate experimental methods for the study of *Bd* must be made so that discoveries leading to understanding and containment of chytridiomycosis can occur.

To date, the methods used to study *Bd* have included those for detection, growth characterization, enzyme production, gene expression, and host response. Detection techniques initially relied upon histology, culture from infected individuals, or electron microscopy, but have advanced to conventional and real-time PCR techniques (Berger et. al., 1998; Berger et. al., 2002; Annis et. al., 2004; Boyle et. al., 2004; Kriger et. al., 2006; Kirshtein et. al., 2007). Growth characterization was performed by observing growth under numerous culture conditions (Piotrowski et. al., 2004; Symonds et. al., 2008). Enzyme activity, specifically of proteases, has been tested using commercial kits or proteolysis observed in culture media containing enzyme substrates (Piotrowski et. al., 2004; Symonds et. al., 2008). Gene expression of stage-specific *Bd* has been performed using RNA microarrays based on the sequenced genome of *Bd* (Rosenblum

et. al., 2008). In comparison to fundamental mycological aspects, the amphibian host response to *Bd* has received more attention from researchers. Amphibian antifungal and antimicrobial agents were studied for their pharmaceutical properties prior to the identification of *Bd* (Clarke, 1997). These studies provided information that served as a precedence for research on innate immune defenses of amphibians to *Bd*. Host peptides are studied by testing their Minimum Inhibitory Concentrations (MICs) when incubated in culture with *Bd* (Rollins-Smith et. al., 2002). RNA microarrays of tissues in amphibians exposed to *Bd* have been used to determine gene expression in response to infection (Ribas et. al., 2009).

Further experiments into the biological nature of *Bd* are needed to pinpoint key cellular components and their functions throughout the life cycle. A single gene regulation study provided information on stage-specific gene regulation in *Bd*, but only with *in vitro* cultures (Rosenblum et. al., 2008). The benefit of having a fully sequenced genome of *Bd* available has been inadequately utilized in studies of gene expression and regulation, interference RNA (iRNA) experiments, and other studies requiring a sequenced genome. Once molecular targets necessary for amphibian infection are identified, cellular localization and tracking can be accomplished by use of fluorescent microscopy targeted to defined macromolecules. Chemotaxis of *Bd* zoospores in response to specific stimuli needs to be more thoroughly tested before it can be confirmed or denied.

The host response to infection has been characterized as consisting primarily of antimicrobial peptides as well as antibodies that act defensively against *Bd* (Rollins-Smith et. al., 2002; Ramsey et. al., 2010). Antimicrobial peptides are released by amphibians following alarm, injury, or stress responses (Gibble et. al., 2008). Correlation between a species' susceptibility to chytridiomycosis and the *in vitro* inhibitory activity of their skin peptides against *Bd* has been demonstrated (Woodhams et. al., 2006; Woodhams et. al., 2007; Rollins-Smith et. al., 2009a). The immune system of *Xenopus laevis* has been well studied as a model for use in developmental, comparative, and evolutionary studies, allowing for easier transition to the study of the immune

response (Du Pasquier et. al., 1989; Du Pasquier et. al., 2000; Robert et. al., 2009). Anti-*Bd* immunoglobulins including IgM, IgX, and IgY were produced and observed in the serum and mucus of *X. laevis* following infection or immunization (Ramsey et. al., 2010). While gene expression patterns indicated no stimulation or suppression of the adaptive immune system in the susceptible *Silurana* (*Xenopus*) *tropicalis*, the presence and concentration of anti-*Bd* antibodies in susceptible species of amphibians has not been described (Rosenblum et. al., 2008). Absence of *Bd*-specific antibodies or cell-mediated responses could provide another indication on the disparities of *Bd* susceptibility among amphibian species. The effect of normal bacterial species on amphibian skin bacteria and their effect on *Bd* infection success remains poorly defined; however, some bacteria have been isolated and found to inhibit *Bd* growth *in vitro* (Rollins-Smith, 2009b).

As detection of *Bd* in an environment or amphibian is pivotal to tracking and diagnosing chytridiomycosis, methods for detection should be readily available, inexpensive, and accurate. The current gold standard method for detection is use of PCR. The conventional method of PCR generates data on the presence or absence of *Bd*, while real-time PCR (RT-PCR) provides quantitative data on *Bd* concentrations. The high cost associated with RT-PCR can prohibit its use when large sample sizes are needed or funding is limited. The QC-PCR method described in Chapter 2 would eliminate the need for a RT-PCR thermal cycler and reduce expenditures relating to collecting quantitative data on *Bd* detection. Diagnosis of chytridiomycosis outside of laboratory settings is currently limited to observation of clinical signs in amphibians. Development and production of a rapid diagnostic test would allow for identification of infected individuals or habitats, providing onsite results while minimizing the need to transport samples to a diagnostic laboratory.

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Presentations

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